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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of BAR-SHAVIT, RACHEL

Serial No. 09/744,679 Group Art Unit: 1635

Filed: 04/11/2001 Examiner: Lacourciere, K.A.

For: **METHOD FOR TREATMENT OF INVASIVE CELLS**

DECLARATION
under Rule 132

Commissioner of Patents and Trademarks
Washington, D.C. 20231

I, Rachel Bar Shavit, an Israeli citizen residing at Ramat Sharet, Jerusalem, Israel, hereby declare:

1. I am currently a Research Group Team Leader (Senior Lecturer) in the Departments of Experimental Medicine & Cancer Research and of Oncology, Hadassah-Hebrew University Hospital, Jerusalem, Israel.

2. My *Curriculum Vitae* and list of publications is attached herewith as Annex "A". My fields of expertise include biology of tumors and angiogenesis.

3. I am the inventor of U.S. Patent Application No. 09/744,679 (hereinafter *"the application"*). The application describes the use of an antisense molecule comprising a nucleotide sequence which is complementary to an RNA sequence of a PAR protein for treating metastatic tumor cells (hereinafter *"the invention"*).

4. I am also familiar with the comments of the examiner in the office action dated February 17, 2004 (hereinafter *"the office action"*).

5. In order to demonstrate that at the time of the invention there was sufficient guidance in the scientific literature to the expression for therapeutic purposes of heterologous nucleic acids, and in order to demonstrate that the state of the art included publications which demonstrated the expression of heterologous sequences in cells and in particular anti-sense therapeutics, I hereby include a list of relevant publications, and a short summary of their subject matter all preceding the date of the application:

- Alama A, Barbieri F, Cagnoli M, Schettini G. **Antisense oligonucleotides as therapeutic agents.** *Pharmacol Res.* 1997 Sep;36(3):171-8.

- Peng H, Callison DE, Li P, Burrell CJ. **Enhancement or inhibition of HIV-1 replication by intracellular expression of sense or antisense RNA targeted at different intermediates of reverse transcription.** AIDS. **1997** Apr;11(5):587-95.

- Hirota J, Baba M, Matsumoto M, Furuichi T, Takatsu K, Mikoshiba K. **T-cell-receptor signalling in inositol 1,4,5-trisphosphate receptor (IP3R) type-1-deficient mice: is IP3R type 1 essential for T-cell-receptor signalling?** *Biochem J.* 1998 Aug 1;333 (Pt 3):615-9.

- Freeman JW, Strodel WE, McGrath PC. **Efficacy of p120 Antisense-Mediated Therapy for Pancreatic Cancer.** *J Gastrointest Surg.* 1997 Oct;1(5):454-460.

- Panegyres PK, Hughes J. **The anticonvulsant properties of antisense c-fos oligodeoxynucleotides in kainic acid-induced seizures.** *J Neurol Sci.* 1997 Dec 9;153(1):12-9.

- Balaji KC, Koul H, Mitra S, Maramag C, Reddy P, Menon M, Malhotra RK, Laxmanan S. **Antiproliferative effects of c-myc antisense oligonucleotide in prostate cancer cells: a novel therapy in prostate cancer**. *Urology.* 1997 Dec;50(6):1007-15.

- Sibille E, Sarnyai Z, Benjamin D, Gal J, Baker H, Toth M. **Antisense inhibition of 5-hydroxytryptamine2a receptor induces an antidepressant-like effect in mice.** *Mol Pharmacol.* 1997 Dec;52(6):1056-63.

6. In order to demonstrate that the anti-sense molecule of the invention is also effective *in vivo*, and in order to demonstrate that it is effect *in vitro* against additional cancers, I hereby enclose the following experiments which were either conducted by me or under my supervision:

7. **Additional *in vitro* results with other cancers**.

Experimental:

Matrigel Assay Invasion: Blind well chemotaxis chambers with 13 mm diameter filters were used for this assay. Polyvinylpyrrolidone-free polycarbonate filters, 8 mm pre size (Costar Scientific Co., Cambridge, MA), were coated with basement membrane Matrigel (25 mg/filter). Briefly, the Matrigel was diluted to the desired final concentration with cold, distilled water, applied to the filters, dried under a hood, and reconstituted with serum-free medium. Cells (2-3x105), suspended in DMEM containing 0.1% bovine serum albumin were added to the upper chamber. Conditioned medium of 3T3 fibroblasts was applied as a chemoattractant and placed in the lower compartment of the Boyden chamber. Assays were carried out at 37°C in 5% CO_2. Over 90% of the cells attached to the filter after a 2h incubation. At the end of the incubation, the cells on the

upper surface of the filter were removed by wiping with a cotton swab. The filters were fixed in methanol and stained with hematoxylin and eosin. Cells in various areas of the lower surface were counted and each assay was performed in triplicate. For chemotaxis studies, filters were coated with collagen type IV alone (5mg/filter) to promote cell adhesion. Cells were added to the upper chamber and conditioned medium was applied to the lower compartment.

Construct:

***hPar1* antisense construct:** *hPar1* cDNA in the antisense orientation. 462 base pairs. as follows:

5'-
CAGGATGAACACAACGATGGCCATGATGTTTAGTGGGAGGCTGACTACAAACA
CTCCGGTGTACACAGATGGGACAAAGAGTGTCAGCCAGGAGCTGGTCAAATATC
CGGAGGCATCTTCTGAGATGAATGCAGGAAGTTGTTTTTGAAGAGGACTGCTTT
TATTGATGGAGACTAATCTGTATTCAGTTAACCCACTTTCATTTTTCTCCTCATCC
TCCCAAAATGGTTCATATTTATCATTGGGGTTCCTGAGAAGAAATGACCGGGGA
TCTAAGGTGGCATTTGTTGCTTTTGATTCTGGCCTGCGGGCCCGGGTGCGGGCAG
ACAACAGCGGGCCGCACAGACTGAAGCAGGCGGCCACCAGCAGCAGCCGCCGC
GGCCCC**CAT**TGTCCCGGGCTCTGCGCGGCGCTGCTCCGGGAGGCTGCCCCGCCTC
GGGCTGCTCCGCTTCACCCTCTCTCCTGA **-3'**

was inserted into the eukaryotic expression plasmid pCDNA3 (Invitrogene, Carlsbad, California) at the *Hind III* and *EcoRI* sites.

Antisense- *hPar1* cDNA was used either in transient transfection experiments or in stable expressing clones as follows:

Antisense- *hPar1* stable clones: Cells (A375SM melanoma) were grown to 30-40% confluence and then transfected with 0.5-2 μg/ml plasmid DNA in Fugene 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. After 10 days of selection, stable, transfected clones were established in medium containing 400-600 μg/ml G418. Antibiotic-resistant cell colonies were transferred to separate culture dishes and were grown-maintained for long term periods in 200 mg/ml G418 medium.

For *hPar1* antisense we have selected clones 3 and clone 4 showing markedly reduced levels of *hPar1* as compared with parental A375SM cells (as outlined in Figure 2d in Even-ram *et al., J. Biol. Chem.* 276(14):10952-10962, 2001).

In order to examine the impact of *hPar1* gene neutralization/silencing, we have performed *in vitro* Matrigel invasion as outlined above.

Results:

The results are shown in Fig. 1(a), 1(b) and 1(c) showing the amount of control (without antisense) melanoma cells (SM), versus the SM transfected with the antisense (SM-AS) in FACS analysis (1a), microscope picture (1b) of the invasion of conrol versus antisense transfected cell; and graph (1c) showing the number of cells that invaded the matrigel coated filteres .

As can be seen from these results the clones exhibiting AS *hPar1* exhibited minimal number of cells capable of invading the Matrigel coated filters as compared with a significant number of cells invading the filters of the parenteral A375 SM cells (Fig. AS melanoma).

These *in vitro* results for melanoma cells together with the results concerning breast cancer cells, already present in the application (Example 3) demonstrate that the antisense technology can inhibit invasiveness of a number of different tumor types.

8. ***In vivo* studies**:

Experimental

Plasmid

The same plasmid was used in the application and as described above (paragraph 7) was used for *in vivo* studies.

Tumorigenicity Studies: Cells from exponential culture of control, and AS transfected *hPar1* clone 3 were detached with trypsin, washed with PBS and brought to a concentration of $1x10^7$ cell/ml. Cell suspension ($2x10^6$/0.2 ml) was inoculated s.c. at the right flank of 5 week-old athymic nude mice CD1-W (Harlan, Jerusalem, Israel). Xenograft size was determined every 3 days by externally measuring tumors in two dimensions using a caliper. Tumor volume was determined by the equation V=LxW2x0.5, where L is the length and W is the width of xenografts. At the end of the experiments, mice sacrificed by cervical dislocation, xenografts were resected, weighed, and fixed in formalin. Paraffin embedded 5- μm sections were stained with H&E or immunostained with anti-PAR1, anti VEGF, and anti-PECAM-1 antibodies using the envision kit according to the manufacturer's instructions (Dako, Glostrup, Denmark).

The results are shown in Fig. 2a, 2b and 2c. Fig 2a shows the pictures (and enlargement) of control mice injected with the tumor cells without antisense construct 4 weeks after injection. Fig. 2b shows the results for mice treated with *hPar1* AS, having the antisense molecule of the invention after the same time period. Fig 2c shows the difference in the tumor size between the control (SM) and treatment (AS).

As can be seen a significant difference was observed between the two groups. All 5 mice in control group regardless either injected with of empty vector cells or injected with non treated tumor cells developed significant tumor mass (see Fig. 2a). In comparison the mice injected AS *hPar1* clones, having the antisense molecule of the invention, consistently either did not develop tumor at all or developed tumor of a minimal size (see Fig. 2d).

9. The above *in vivo* results show that antisense molecule of the invention was effective in an *in vivo* setting, inside a body of an animal.

10. The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: June 10/04

Rachel Bar Shavit

Dr. Rachel Bar Shavit

hPar1 antisense inhibits melanoma cell (SM-A375) invasion *in vitro*

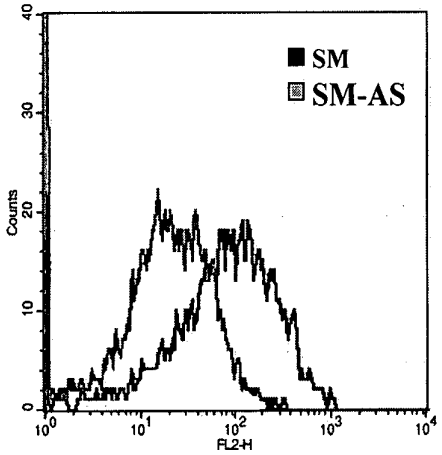


Fig 1(a)

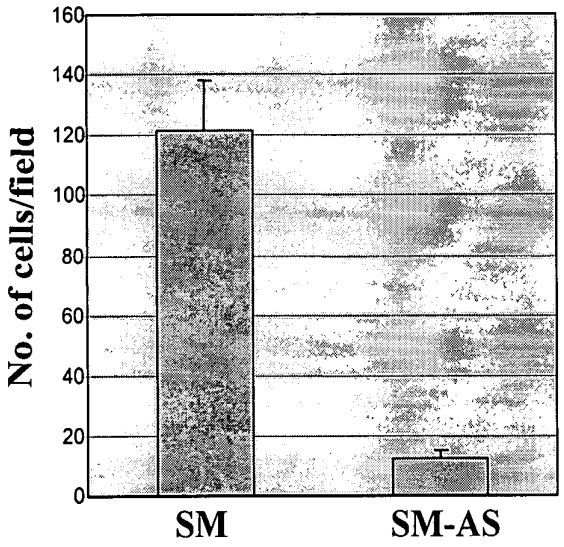


Fig 1(c)

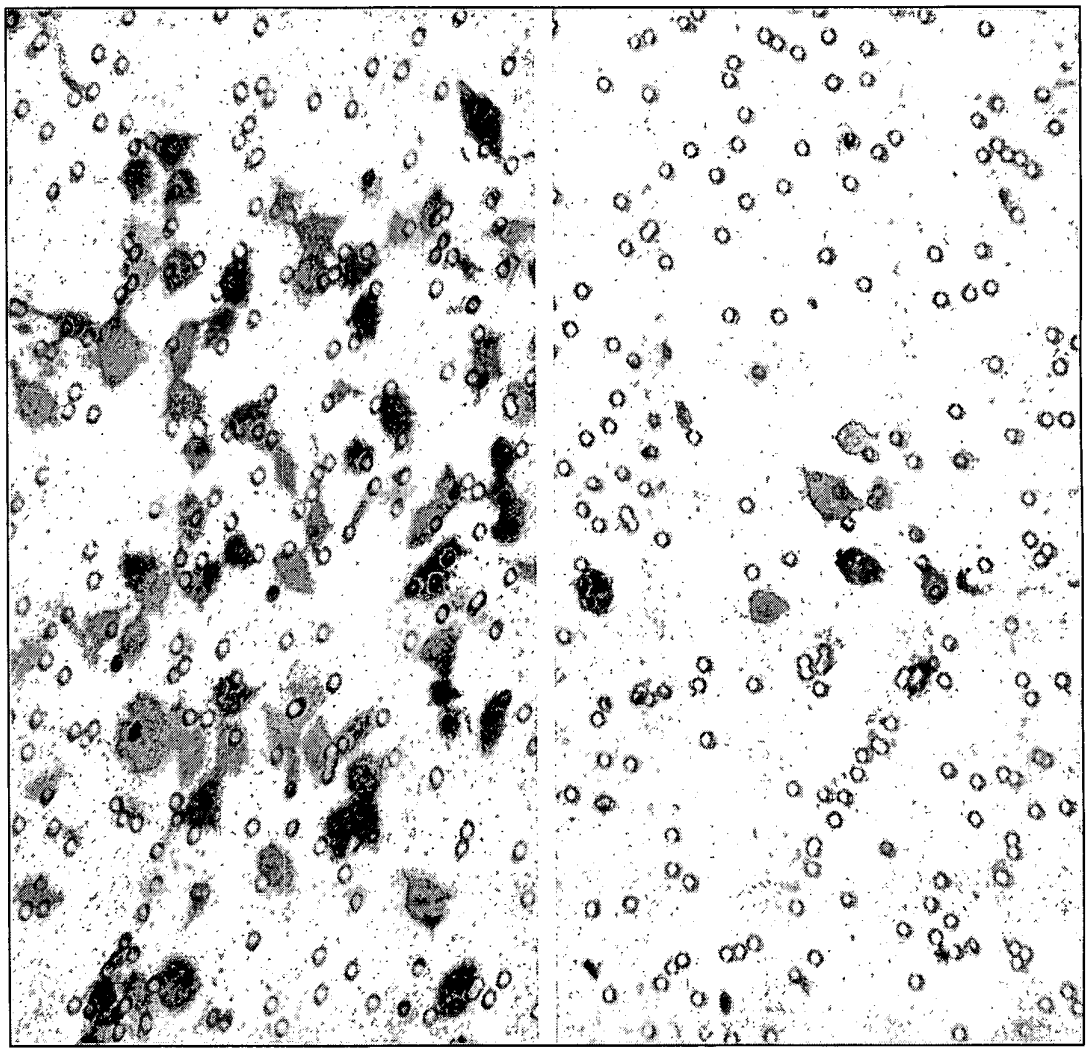
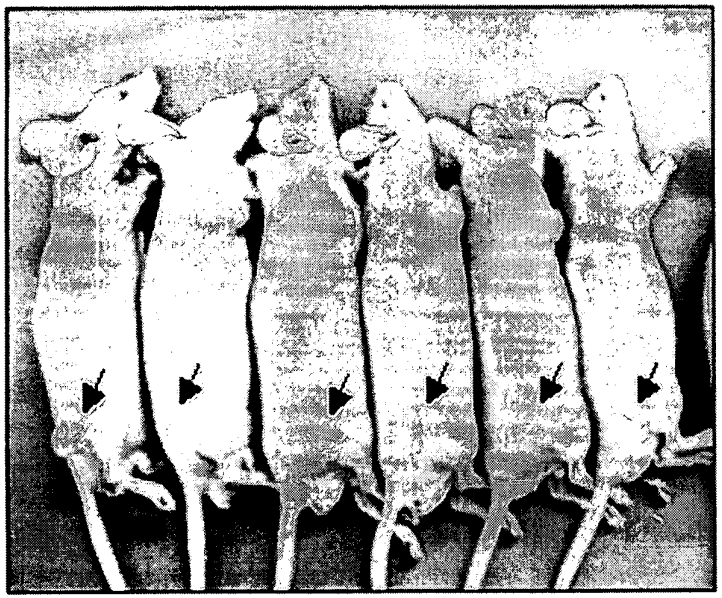


Fig 1(b)

Fig 2(a)

SM



SM

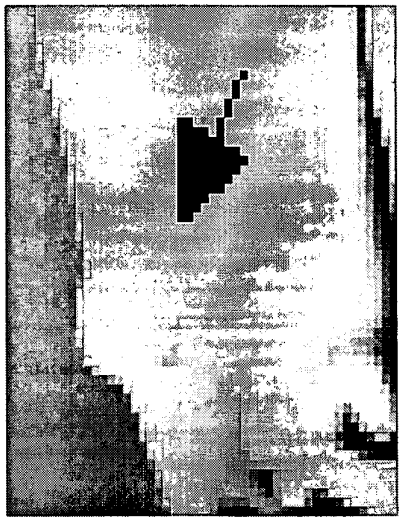
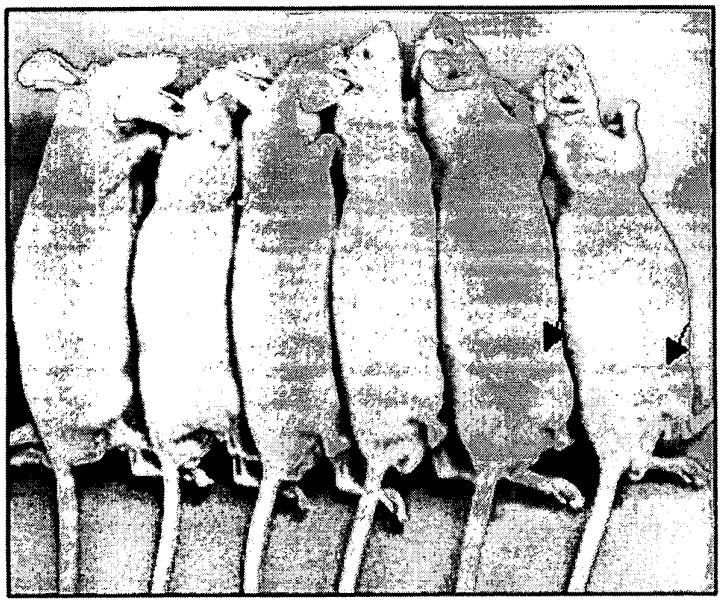


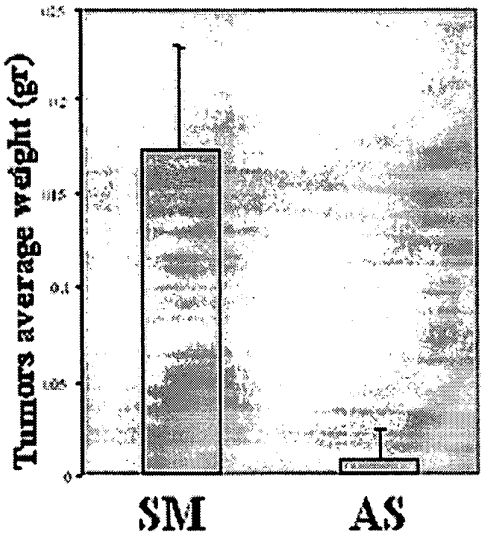
Fig 2(b)

AS



AS

Fig 2(c)



Annex A

CURRICULUM VITAE

1. Personal Details:

Name:	Rachel Bar-Shavit, Ph.D.
Date of Birth:	December 1, 1953
Country of Birth:	Hadera, Israel
Identity Number:	5211059
Marital Status:	Married, two children
Home Address:	Peretz-Bernstein 44, 96920 Ramat Sharett, Jerusalem
Phone:	02-6777563 (W) 051-874132 02-6414188 (H)
Fax:	02-6422-794
e-mail:	barshav@md.huji.ac.il

2. Education:

1971-1974	B.Sc., Cum laude, Bar-Ilan University, Ramat-Gan, Israel.
1974-1977	M.Sc. Life Sciences, The Feinberg Graduate School, Weizmann Institute of Science, Rehovot, Israel. Instructor: Prof. Yosef Aloni, Department of Genetics Thesis: "Regulation of Transcription in Polyoma Virus"
1978-1982	Ph.D., Life Sciences, Bar-Ilan University. Instructor: Dr. Asher Shainberg Thesis: "Studies on Acetyl Choline Receptor Ssynthesis and Characterization *In Vitro*"
1982-1985	Postdoctoral Fellow, Department of Pathology, Washington University School of Medicine and The Jewish Hospital of St. Louis, St. Louis, Missouri. Instructor/Host: Dr. George D. Wilner Research Topic: "Thrombin interaction with monocyte/macrophages."
1985-1986	Research Associate in Pathology, Department of Pathology, Washington University School of Medicine and The Jewish Hospital of St. Louis, St. Louis, Missouri.

3-6. Professional Experience:

1996 - present	Senior Lecturer, Department of Experimental Medicine & Cancer Research and Department of Oncology, Hadassah-Hebrew University Hospital, Jerusalem.
2001	Sabbatical at Harvard University, Dept. of Cell Biology
1990-1995	Lecturer, Department of Experimental Medicine & Cancer Research and Department of Oncology, Hadassah-Hebrew University Hospital, Jerusalem.
1987-1989	Research Associate in Oncology, Department Oncology, Hadassah - Hebrew University Hospital, Sharett Institute, Jerusalem.

Honors:

1983: Postdoctoral Fellowship Award, American Heart Association

1990: The Hebrew University, Faculty Prize in memory of Prof. Gabriel Issac- Distinguished Research

1992: The Sanofi Foundation for Thrombosis Research, Scholarship for a Young Academic Research in Hemostasis, Inflammation and Immunology

1994: The Hebrew University, Faculty Prize for Distinguished Research Project

1999: "The Israel Cancer Association"- the 50th Anniversary Grant Award

2001: " The George and Eva Klein" price, for distinguished research- proposal in tumor biology - ISF, Israel Scientific Fund (Academia).

Faculty Activities: 1997-2000 Committee "Vaadat Kabala" Medical School

8. Teaching at the Hebrew University:

* See attached relevant forms

Research Group is focusing on:

"The Role of PAR family in Tumor Invasion , Metstasis & Angiogenesis "

Interactions with clinician (i.e. Dr. Uziely Beatrice, Head of Ambulatory Service Unit Oncology, Dr. Grisaru-Granovsky, S., Gynecologist, Drs. Diana Prus and Dr Galina Pizov Pathologist).

Students in the lab: ***Even-Ram, Sharona*** *Ph.D. (currently a Post Doc fellow at NIH under the supervision of Ken Yamada)*

MSc. students: - **Efrat landau** M.Sc. completed 1999, Recipient of Faculty Research Award on Thesis)
-**Vered Katz,** Beginning: 2002/2003

Current Ph.D. students: **Yin Yong Jun** (establishing *hPar1* transgenic mouse model targeted to the mammary glands)

Zaidoun Salah (Subject: Hormone regulation of *hPar1: Identification of ARE in the promoter)*

Grisaru-Granovsky, Sorina (I. The role of *hPar1* in gestational trophoblastic diseases (GTD): Normal and pathological placenta, II. Differential expression of *hPar1* in ovary malignant progression)

Hagit Turm (*hPar1* and development)

Irit Cohen (in collaboration with Prof. Israel Vlodavsky); *hPar1* Signaling

MD student: * *Idit Pazgal* (Recipient of Faculty Research Award on Thesis)
**Pokroy Elisheva* (Recipient of Faculty Research Award on Thesis)

Research Technician: **Maoz Miriam**

List of Publications

1. **Bar-Shavit, R.,** Loub, O., and Aloni, Y. The frequencies of transcription from the E- and L- strand of Polyoma DNA. ***J Gen. Virol.*** 39: 357-360, 1978.

2. Kaufmann, G., **Bar-Shavit, R**. and DePamphilis, M.L. Okazaki pieces grow opposite to the replication fork direction during Simian virus- 40 DNA replication. ***Nucleic Acids Res.*** 5: 2535-2545, 1978.

3. DePamphilis, M.L., Anderson, S., **Bar-Shavit, R**. et al. Replication and structure of Simian virus 40 chromosomes. ***Cold Spring Harbor Symp***. 43: 679-692, 1978.

4. **Bar-Shavit, R.,** Kahn, A., Fenton II, J.W. and Wilner, G.D. Chemotactic response of monocytes to thrombin. ***J Cell Biol***. 96: 282-285, 1983.

5. **Bar-Shavit**, R., Kahn, A., Wilner, G.D. and Fenton II, J.W. Monocyte chemotaxis: stimulation by specific exosite region in thrombin. ***Science*** 220: 728-731, 1983.

6. **Bar-Shavit, R.,** Kahn, A., Fenton II, J.W. and Wilner, G.D. Receptor-mediated chemotactic response of macrophages to thrombin. ***Lab. Invest.*** 49: 702-707, 1983.

7. Shainberg, A., Brik, H., and **Bar-Shavit, R.** Effect of thyroid hormones on acetylcholine receptors and Na-K-ATPase in muscle cultures. In: Experimental Biology and Medicine, vol. 9, pp. 30-33, Krager, Basel, 1984.

8. Shainberg A, Brik H, **Bar-Shavit R,** Sampson SR. Inhibition of acetylcholine receptor synthesis by thyroid hormones. ***J Endocrinol***. May;101(2):141-7, 1984.

9. **Bar-Shavit, R.,** Kahn, A., Wilner, G.D., Mann, K.G. and Fenton II, J.W. Thrombin chemotactic domain is licalized within a B chain CNBr fragment. ***Biochemistry***. 23: 397-400, 1984.

10. **Bar-Shavit R**, Wilner GD. Mediation of cellular events by thrombin. ***Int Rev Exp Pathol.*** 29:213-41, 1986.

11. **Bar-Shavit, R.,** Kahn, A.J., Mann, K.G. and Wilner G.D. Identification of a thrombin sequence with growth factor activity on macrophages. ***Proc. Natl. Acad. Sci.*** USA 83: 976-980, 1986.

12. **Bar-Shavit, R.,** Hruska, K., Kahn, A.J. and Wilner, G.D. Hormone -like activity of human thrombin. ***Ann. N.Y. Acad. Sci.*** 485: 335-348, 1986.

13. **Bar-Shavit, R.** and Wilner. G.D. Biological Activities of non-enzymatic thrombin: elucidation of a macrophage interactive domain. ***Sem. Thromos. Haemostas.*** 12: 244-249, 1986.

14. **Bar-Shavit, R.,** Kahn, A.J., Mann, K.G. and Wilner, G.D. Growth promoting effects of esterolytically inactive thrombin. ***J Cell Biochem.*** 32: 261-272, 1986.

15. **Bar-Shavit, R.,** Hruska, K.A., Kahn, A.J. and Wilner, G.D. Thrombin chemotactic stimulation of HL-60 cells. Studies on thrombin responsievness as a fuction of differentiation. ***J Cell Physiol.*** 131: 255-261, 1987.

16. Spira, O., Atzmon, R., **Bar-Shavit**, R., Gross, J., Gordon, A. and Vlodavsky, I. Striated muscle fibers differentiate in primary culutes of adult anterior pituitary cells. ***Endocrinology.*** 122: 300-304, 1988.

17. **Bar-Shavit,** R., Eldor, A. and Vlodavsky, I. Binding of thrombin to subendothelial extracellular matrix: Protection and expression of Functional properties. ***J Clin. Invest.*** 84: 1096-1104, 1989.

18. Vlodavsky I, Korner G, Ishai-Michaeli R, Bashkin P, **Bar-Shavit R**, Fuks Z. Extracellular matrix-resident growth factors and enzymes: possible involvement in tumor metastasis and angiogenesis. ***Cancer Metastasis Rev.*** Nov;9(3):203-26, 1990.

19. Chajek-Shaul, T., Friedman, G., Bengtsson-Olivecrona, G., Vlodavsky, I. and **Bar-Shavit, R.** Interaction of lipoprotein lipase with subendothelial extracellular matrix. ***Biochem. Biophysica. Acta.*** 1042: 168-175, 1990

20. **Bar-Shavit, R.,** Benezra, M., Eldor, A., Hy-Am, E., Fenton II, J.W., Wilner, G.D. and Vlodavsky, I. Thrombin immobilized to extracellular matrix is a potent mitogen for vascular smooth muscle cells: Non-enzymatic mode of action. ***Cell Regulation.*** 1: 453-553, 1990.

21. Vlodavsky I, Bashkin P, Ishai-Michaeli R, Chajek-Shaul T, **Bar-Shavit R**, Haimovitz-Friedman A, Klagsbrun M, Fuks Z. Sequestration and release of basic fibroblast growth factor. ***Ann N Y Acad Sci.***;638:207-20, 1991, review.

22. **Bar-Shavit, R.,** Sabbah, V., Lampugnany, M.G., Marchisio, P.C., Fenton II, J.W. Vlodavsky, I. and Dejana, E. An Arg-Gly-Asp sequence within thrombin promotes endothelial cell adhesion. ***J Cell Biol.*** 112: 335-445, 1991.

23. Vlodavsky I, **Bar-Shavit R**, Ishai-Michaeli R, Bashkin P, Fuks Z. Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? ***Trends Biochem Sci.*** Jul;16(7):268-71, 1991, Review.

24. Vettel, V., **Bar-Shavit, R.,** Simon, M.M., Bruner, G., Vlodavsky, I. and Kramer, M.D. Coexpression, coordinate secretion and synergistic extracellular activity of T-cell associated serine proteinase-1 (MTSP-1) and endoglycosidic enzyme(s) of activated T-cells. ***Eur. J Immunol.*** 21: 2247-2251, 1991.

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26. Vlodavsky I, Ishai-Michaeli R, Mohsen M, **Bar-Shavit R**, Catane R, Ekre HP, Svahn CM. Modulation of neovascularization and metastasis by species of heparin. ***Adv Exp Med Biol.*** 313:317-27, 1992.

27. **Bar-Shavit, R.,** Benezra, M., Sabbah, V., and Vlodavsky, I. Thrombin as a multifunctional protein: Induction of cell adhesion and proliferation. Update, ***Am. J Respi. Cell Mol. Biol.*** 6:123-130, 1992 Review.

28. Benezra, M., Vlodavsky, I., and **Bar-Shavit, R**. Thrombin enhancement of heparan-sulfate degradation in the subendothelial extracellular matrix by highly metastatic lymphoma cells. ***Exp. Cell Research.*** 201:208-215, 1992.

29. Benezra, M., Vlodavsky, I., Yayon, A., **Bar-Shavit, R.,** Regan, J., Chang, M., and Ben-Sasson, S. Reversal of bFGF-mediated cell transformation by aromatic anionic compounds. ***Cancer Research***. 52: 5656-5662, 1992.

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34. Benezra , M. ,Ben-Sasson, S., Regan, J., Chang, M. , **Bar-Shavit, R**. and Vlodavsky, I. Antiproliferative activity towars vascular smooth muscle cells and receptor binding of hepsrin-mimicking anionic aromatic compounds. ***Arteriosclerosis and Thrombosis***. 14 (12) : 1993-1999, 1994.

35. Herbert, J-M., Dupuy, E., Laplace, M-C., Zini, J-M., **Bar-Shavit, R.** and Tobelem, G. Thrombin induces endothelial cell growth via both proteolytic and a non-proteolytic pathway. ***Biochem. J***. 303:227-231,1994.

36. **Bar-Shavit, R.** , Ginzburg, Y., Maoz, M., Vlodavsky, I. and Peretz, T. The involvement of thrombin - RGD in metastasis: Characterization of a cryptic adhesive site. ***Israel J. Med***. 31: 86-94, 1995.

37. Bitan, M., Mohsen, M., Levi, E., Wygoda, M., Miao, H-Q., Lider, O., Svahn, C.M., Ekre, H. P., Ishai-Michaeli, R., **Bar-Shavit, R.,** Vlodavsky, I. and Peretz, T. Structural requirement for inhibition of melanoma lung colonization by heparanase inhibiting species of heparin. ***Thrombosis and Hemostasis***. 31: 106-118, 1995.

38. Vlodavsky I, Miao HQ, Atzmon R, Levi E, Zimmermann J, **Bar-Shavit R,** Peretz T, Ben-Sasson SA. Control of cell proliferation by heparan sulfate and heparin-binding growth factors. ***Thromb Haemost***. Jul;74(1):534-40, 1995.

39. **Bar-Shavit, R.,** Maoz, M., Ginzburg, Y. and Vlodavsky, I. Specific involvement of glypican in thrombin adhesive properties. ***J. Cell Biochem***.61: 278-291, 1996.

40**. Even-Ram, S., Uziely, B., Cohen, P., Grisaru-Granovsky, S., Ginzburg, Y., Maoz, M., Ginzburg, Y., Reich, R., Vlodavsky , I., and **Bar-Shavit, R.** Thrombin receptor overexpression in malignant and physiological invasion processes. ***Nature Medicine. 4: 909-914,1998.

41. Landau, E. Tirosh, R. Pinson, A. Banai, S., Even-Ram, S. Maoz, M. Katzav, S. and **Bar-Shavit, R.** Protection of thrombin receptor expression under hypoxia. ***J. Biol. Chem***. 275: 2281-2287, 2000.

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ANTISENSE OLIGONUCLEOTIDES AS THERAPEUTIC AGENTS

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The potential for modulating gene expression by the use of antisense oligonucleotides has become increasingly interesting in recent years.

Antisense oligonucleotides are complementary nucleic acid fragments that hybridize to target sequences within RNA to form a DNA-RNA duplex, resulting in the block of translation of messenger RNA into the protein. Advances in chemistry and molecular biology have provided the basis to develop antisense oligodeoxynucleotides and improve their selectivity, stability and specificity of action. The antisense technology has been extensively used *in vitro* and *in vivo* as a tool to study the regulatory mechanisms in biologic processes and as potential therapeutic agents in cancer, viral infections and genetic disorders.

In the present review, the various approaches for the use of antisense molecules in oncology, virology, genetic and inflammatory diseases are described; several studies, supporting the *in vitro* and *in vivo* applications of this technology, are also presented. Moreover, the potential clinical use of antisense therapies is discussed.

KEY WORDS: antisense oligonucleotides, gene expression, oncogenes, viral targets.

INTRODUCTION

The classical approach for discovering new drugs involves the use of massive screening programmes. An alternative strategy is to use the information content of nucleic acids as the basis for a new class of DNA therapeutic compounds. The potential to inhibit the genes that are implicated in malignant and viral diseases is indeed very attractive. Advances in biotechnology have now enabled the synthesis of short nucleic acid fragments, called oligonucleotides, complementary or 'antisense' to a specific target sequence, able to interfere with gene function through the hybridization with their specific mRNAs. Upon binding to the RNA target, the antisense oligomers can block the translation and inhibit the production of the protein.

Synthetic oligonucleotides (ODNs) hybridize specifically to complementary nucleic acid through hydrogen bonding, usually Watson–Crick base pairing. Since, statistically, the minimum number of nucleotides that occurs just once in the human genome is, on average, 13, antisense ODN (aODN) longer than this (15–20 mer) should be able to bind selectively to a unique RNA species in the cell. Thus, a single mismatched base could consistently decrease the affinity and specificity of the aODN.

STABILITY

One of the basic requirements to be met by aODN, besides specificity, is stability, both under assay conditions and *in vivo*, following therapeutic use. Oligonucleotides, as any other type of drug, can exert their activity only by encountering the following criteria: a sufficient intracellular concentration; a sufficient intracellular half-life; serum, body

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fluids and intracellular nuclease stability; and an efficient cellular uptake and distribution.

Indeed, naturally occurring DNA is particularly sensitive to enzymatic degradation by exo- and endo-nucleases, *in vivo* and *in vitro* (serum-containing medium). To overcome this problem, chemically modified nuclease-resistant oligonucleotide analogs have been developed. All the three structural elements of ODNs could be modified, i.e. the base, the phosphodiester group and the sugar. Nevertheless, the most notable analogs are modified at the phosphate backbone, where the nucleolytic attack occurs through the replacement of an oxygen atom by a sulphur atom (phosphorothioates) or a methyl group (methylphosphonates). Perhaps the greatest success in oligo modification has been achieved with the phosphorothioate ODNs (PS-ODNs) which met most of the criteria for clinical therapeutic use, i.e. water solubility, stability to nuclease cleavage and easy synthesis in bulk.

UPTAKE, INTERNALIZATION AND MECHANISMS OF ACTION

The ability of oligos to cross cellular membranes efficiently is a critical point in developing these agents as therapeutics. Carrier systems for increasing cellular uptake and delivery of the aODNs to the target mRNA site, have been reported such as the conjugation to cholesterol, poly-L-lysine, transferrin-poly-L-lysine and the incapsulation in cationic or antibody-targeted liposomes [1–3]. A paper by Aoki *et al.* [4] demonstrated that the liposome-mediated *in vivo* gene transfer of aODN against the K-*ras* construct may be a useful therapeutic strategy for aODN delivery. Indeed, the growth of pancreatic cells (with K-*ras* point mutation) was reduced by aODN to K-*ras* in culture tests. Moreover, nude mice inoculated with these cells and treated with aODN-liposome complex, did not show peritoneal tumour dissemination.

Controversy still exists over the processes of internalization: it has been hypothesized that it is: (1) a fluid-phase pynocytosis; (2) an active transport (calcium-dependent) of the charged ODN, by endocytosis, which is receptor-mediated; or (3) a passive diffusion of the uncharged oligomer. The entry of phosphodiester and phosphorothioate ODNs by endocytosis occurs by a saturable process in which the cell membrane encloses the ODN giving rise to a small vescicle that is addressed to the cytoplasm. On the contrary, methylphosphonate ODNs are mainly taken up by a fluid phase process. Once inside the cell, the aODN binds mRNA to create a double helix DNA/RNA that interferes with the translational process. Translation is carried out by structures called ribosomes, which construct nascent proteins 'reading' RNA trancripts. The binding of the aODN to mRNA can inhibit translation by interrupting ribosome activity. Another mechanism of action, by which translational inhibition may occur, is mediated by the induction of ribonuclease H (RNAse H) that cleaves the mRNA at the site of ODN binding. RNAse H is a ubiquitous enzyme which catalyses the hydrolysis of the RNA strand in a DNA/RNA heteroduplex. The target mRNA sequence is inactivated as an irreversible event since the antisense DNA may degrade several mRNA molecules, through separate hybridizations, resulting in the dissociation of cleaved products.

To summarize, aODNs can exert their activity through at least three mechanisms: (1) a steric hindrance, blocking pre-mRNA function or interacting

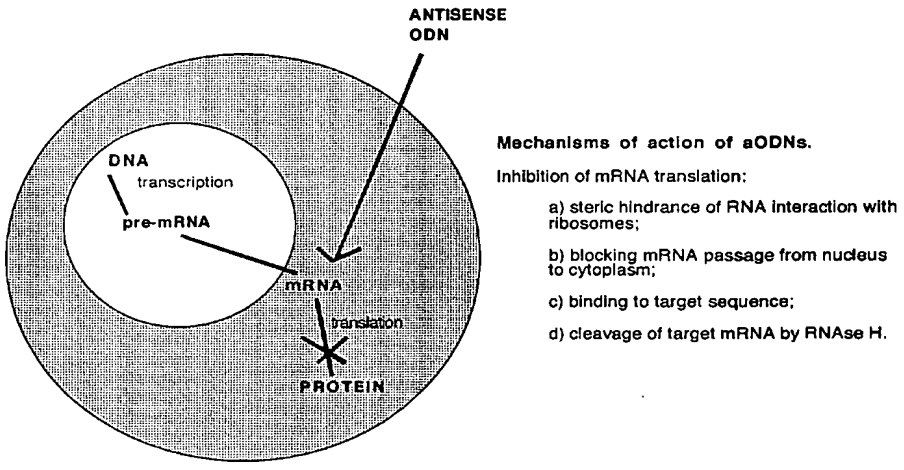

Fig. 1.

with ribosomes; (2) a specific hybridization with the cytoplasmic target mRNA sequence, resulting in the arrest of translation; and (3) a cleavage of target mRNA by the endogenous enzyme RNAse H (Fig. 1). To date, however, the exact molecular pathways, over which the inhibition of targeted gene expression is mediated, are still under investigation.

PHARMACOKINETICS

Preliminary pharmacokinetic studies, carried out with the PS-ODN in mice model, showed that aODN administered in non-toxic doses is excreted in the urine over 2–3 days with an average half-life of 20–40 h after intravenous (i.v.) injection [5]. In addition, a more recent study by Agrawal *et al.* [6] describes absorption, distribution and *in vivo* stability of a 25-mer ^{35}S-labelled ODN in rats by oral administration (50 mg kg^{-1}). Stable ODN was detected in the gastrointestinal tract for up to 6 h and as well as the intact ODN, was in the plasma and various tissues. Degradative and intact products were found in the urine. The oral therapeutic use of oligomers in humans might gain useful indications from the above mentioned study. The pharmacokinetic and biostability of a PS-ODN anti-HIV-I, named gene expression modulator 91 (GEM 91), was determined in rats following i.v. route in a paper by Zhang [7]. Plasma disappearance of the radiolabelled GEM 91 was biphasic with an initial half-life of 0.95 h (distribution out of the plasma compartment) and a second half-life of 45.57 h (elimination from the body). GEM 91 was primarily excreted in urine (27% within 24 h and 58% over 240 h after administration), whereas faecal elimination was a minor excretion pathway. Additionally, a wide tissue distribution (kidney, liver, heart, intestine) of intact GEM 91 was described. This study has provided the basis for a pharmacokinetic analysis of GEM 91 in humans [8]. Six AIDS patients, injected with ^{35}S-labelled GEM 91 (2 h; 0.1 mg kg^{-1} i.v.), showed an extensive tissue distribution of the ODN within 30 min of infusion with a major pathway of elimination (approximately 50% of the administered dose) represented by urinary excretion. Furthermore, a recent paper by Bishop *et al.* [9], describing a phase I trial of a PS-aODN to p53, involving 16 patients with hematologic malignancies, reported that approximately 36% of ODNs was recovered intact in the urine while 56% were highly degraded. Since the plasma aODN concentration was less than 1 μM, the non-specific ODN-related toxicity might allow further increase of the dosage.

The lack of specificity represents the most important limiting factor in the treatment of several pathologic process; although aODNs can selectively target genetic material they can also exhibit a sequence independent activity through the interaction with other cellular macromolecules such as DNA polymerase [10], HIV reverse transcriptase [11], telomerase [12], tyrosine kinase [13]. Immune stimulation *in vivo* has been also demonstrated by antisense oligomers complementary to the *rev* gene of HIV-I [14] and the transcription factor NF-κB p65 [15].

MOLECULAR TARGETS FOR aODNS

Applications of aODN have been directed towards understanding the function of genes or modulating gene expression in viral and cancer diseases in a variety of systems. To date, several investigations have demonstrated that ODNs are able to inhibit the replication of different viruses in tissue cultures, the expression of cellular oncogenes and to control genetic diseases. Encouraging results have been obtained in cell-free protein systems, by administration of the aODNs to the cultures and by direct injection into the cells. For the *in vivo* utilization of oligomers, animal models mimicking human malignancies have been developed. Moreover, clinical trials are currently under investigation according to procedures that can take place directly in the patients (*in vivo*) or in cells obtained from the body (*ex vivo*). More in detail, antisense therapeutic agents are investigated for treating HIV, hepatitis B, herpes simplex and papilloma virus infections, cancer, restenosis, rheumatoid arthritis and allergic disorders.

In this article we review the most recent papers focusing the antisense use in cancer, viral infections and other diseases; a summary of aODN against different targets is also provided in Table I.

CANCER-RELATED TARGETS

Several papers have reported the use of antisense strategy to study normal gene function and block gene expression. In particular, genes responsible for malignancies have been largely employed as suitable targets to assess the activity of aODN as anticancer compounds. The potential application of aODN in cancer treatment relies on the association of oncogenes with human malignancies: the knowledge of the sequences of these genes has provided the essential scientific basis for genetic targeting with aODN.

Among oncogenes, *myc* and the *BCR/ABL* fusion gene have received a great deal of attention in antisense studies. The c-*myc* proto-oncogene plays a central role in the regulation of cell growth and differentiation and alterations of the c-*myc* expression are frequently associated to malignancies of

Table I
Summary of aODN activities

Target	*References*
Oncogenes	
c-*myc*	[17–19)*; [32]†
BCR/ABL	[20]*; [21, 22]†
bcl-2	[24, 25]*; [26]†
p53	[27–29)*; [9]†
c-myb	[30]*; [31]†
Viruses	
HPV	[33]†
HIV-I	[34–36]*; [37, 38]†
CMV	[39, 40]*; [41, 42]†
HBV	[43]†
HCV	[44]*
HSV	[45]*; [46]†
Others	
β-globin	[47]*
Adenosine	[48]†
NF-κB*p65*	[49]†

**In vitro.*
†*In vivo.*

diverse histogenesis [16]. In recent years, aODNs to c-*myc* have been widely investigated as inhibitors of human leukaemia cell line proliferation [17, 18] and to prevent T lymphocytes entering into the S-phase [19], *in vitro*. The *BCR/ABL* gene, resulting from the translocation of the *ABL* proto-oncogene from chromosome 9 to 22 (to the *BCR* site) forming the 'Philadelphia' (Ph) chromosome, is found in patients with hematologic neoplasms. Since *BCR/ABL* is a distinctive feature of chronic myelogenous leukaemia (CML), this gene offers a highly selective molecular target for therapeutic intervention. Indeed, synthetic 18-*mer BCR/ABL* oligomers have been demonstrated to suppress CML blast crisis cell growth in cultures [20]. A more recent study, carried out *in vivo*, reported that a 26-*mer* aODN (1 mg/day for 9 days), administered to immunodeficient (SCID) mice injected with human Ph leukaemic cells, was able to reduce the levels of *BCR/ABL* transcripts in mouse tissues and to prolong the survival of the mice [21]. On this basis, in order to enhance the suppression of *BCR/ABL* oncogene expression and achieve the *in vivo* arrest of the disease progression, the effects of the combined treatment of aODN with a conventional antineoplastic compound have been assessed. Skorski *et al.* [22] evaluated the ability of a 16 *mer BCR/ABL* aODN, subsequent to administration of cyclophosphamide, to retard the leukaemic process induced in SCID mice bearing Ph cells. Results from this study showed that 50% of the mice treated with antisense and cyclophosphamide appeared to be cured of leukaemia: mice were alive and apparently disease free more than 60 weeks after injection of leukaemia cells. Thus, in this experimental model, such an innovative strategy may lead to the clinical setting of combination therapies with the view of reducing drug resistance development and minimizing toxicity.

Hematological malignancies commonly display chromosomal translocations (leading to hybrid genes). Among them the t(14;18) translocation juxtaposes the *bcl-2* gene to the immunoglobulin heavy-chain (IgH) locus increasing the cellular level of *bcl-2* mRNA and protein, responsible for the neoplastic transformation of most of follicular B-cell lymphomas. In addition, since *bcl-2*-related proteins have been shown to control programmed cell death [23], they constitute suitable candidates for antisense-based therapy in several diseases mediated by hyperexpression of *bcl-2* and deregulated cell death [24, 25]. The *in vivo* suppression of B-cell lymphoma with *bcl-2* antisense ODNs have been carried out by Pocock *et al.* [26] in a SCID-B-cell lymphoma mouse model.

Among all the oncogenes involved in solid tumour transformation, *p53* is one of the major ancogenes responsible for the process of cancerogenesis. The wild type tumour–suppressor gene *p53*, is fundamental for normal cell growth and metabolism while its mutated form is the most frequently found in a wide range of tumours. Moreover, the modulation of this tumour suppressor gene represents an attractive target for gene specific therapeutics and for the understanding of dysfunctional gene expression. The role of *p53* has been investigated by an antisense approach in a variety of cancer cell lines such as pancreatic [27], ovarian [28] and colon [29]. In addition, increased levels of the phosphoprotein *p53*, both wild type and mutant, are involved in myelodysplastic syndrome and acute myelogenous leukaemia. A phase-I trial of aODN directed against *p53* mRNA was conducted in 16 patients with advanced hematologic malignancies [9]. Patients were given a 20-*mer* phosphorothioate ODN at doses of 0.05 to 0.25 mg kg^{-1} per hour for 10 days by continuous i.v. infusion. Results demonstrated minimal or non-toxic effects after aODN treatment, however, although leukaemic cell growth *in vitro* was inhibited as compared with pre-treatment samples, there were no clinical complete responses. Nevertheless, this study consistently supported the possibility to administer aODNs systemically to humans, without significant adverse effects.

The *myb* gene has been investigated as a potential target for aODNs. The proto-oncogene c-*myb* regulates cell growth and differentiation in several cell types playing a relevant role in the pathogenesis of malignant transformation (melanoma, leukaemia) and diverse diseases such as atherogenesis. The c-*myb* product is differentially required for the proliferation of both normal hematopoietic progenitors and acute myelogenous leukaemia cells. Antisense

oligomers have been used to determine the role of c-*myb* in chronic myelogenous leukaemia (CML) and to test the antiproliferative activity in mononuclear cells obtained from patients with blast crisis (CMLBC) or chronic phase cells. The inhibition of *in vitro* proliferation of both CML cells by aODNs suggest that the proliferative potential of these progenitors are sensitive to c-*myb* expression [30].

The therapeutic potential of antisense agents has also been investigated in the treatment of cutaneous melanoma, a highly malignant and increasingly common neoplasm. c-*myb* and c-*myc* oncogenes have been shown to be involved in the pathogenesis of melanoma. Using aODNs, a specific decrease of *myb* mRNA and cell growth have been obtained in several human melanoma cell lines. In addition, *in vivo* experiments with SCID mice, bearing human malignant melanoma, demonstrated that tumour growth was suppressed after aODN infusion [31]. A recent paper by Leonetti *et al.* [32] described the antiproliferative effects (> 50% within 24 h of the end of treatment) of 15-*mer* c-*myc* antisense in three human melanoma cell lines overexpressing c-*myc* mRNA *in vitro*. *In vivo* treatment of nude mice, bearing NG (human primary melanoma line) xenograft, with the same aODNs, exhibited antitumour activity by reducing the tumour growth, the number of lung metastasis and increasing lifespan [32].

OLIGONUCLEOTIDES AS ANTIVIRAL AGENTS

Several investigations have suggested *in vitro* and *in vivo* activities of antisense drugs against viral targets. Viruses are one of the best targets for antisense therapeutics because they have proteins with no normal cellular counterpart so that possible interactions with cell functions are reduced.

The human papillomavirus (HPV) is believed to play a relevant role in the etiology of cervical carcinomas (90% contain HPV genes). In particular, the two viral oncoproteins, E6 and E7, which are able to establish and maintain the malignant phenotype, can be specific targets for antisense therapy. The antiproliferative effects of PS oligos, against HPV E6 and E7 mRNAs in cervical cancer lines and primary tumour explants, demonstrated their role in the malignant proliferation of cervical cancer [33].

Nevertheless, the most challenging area of application of aODNs is in the treatment of acquired immunodeficiency syndrome (AIDS). An appropriate target for aODNs has been identified in the *rev* region of human immunodeficiency virus type I (HIV-I), the causative agent of AIDS, being highly conserved and critical for the production of full-length viral transcripts. The earliest work by Matsukura *et al.* [34] reported that a 14-*mer* PS antisense oligo to the HIV-I *rev* mRNA was able to block the cytopatic effects of HIV-I in a T cell-derived line. The antiviral activity of aODN has been studied for HIV-I proteins that regulate viral expression such as *tat* and *rev* [35]. More recently, by taking into account the large amount of encouraging evidence provided by experimental models, PS analogues have been also introduced in AIDS clinical trials but results are still ongoing [36–38].

Inflammation of the retina, caused by cytomegalovirus (CMV), usually affects AIDS patients in the terminal stages of their disease: an antisense compound, complementary to a portion of CMV mRNA, able to arrest virus replication, has been developed by two pharmaceutical companies (ISIS and Eisai). The antiviral activity of the phosphorothioate oligonucleotide ISIS 2922, complementary to the human CMV RNA, has been assayed in U373 cells transformed with CMV cDNA and CMV-infected human skin fibroblasts [39, 40]. The expression of the CMV Immediate-Early 55-kDa protein was reduced in the two cellular systems at nanomolar concentrations. It should be noted that the antiviral activity of ISIS 2922 resulted from three different mechanisms of action: (1) antisense mediated; (2) non-antisense inhibition of virus replication; and (3) sequence-independent inhibition of virus absorption to host cells. The multiple mechanisms of ISIS 2922 may provide advantages for a therapeutic use, preventing development of resistance. This ODN has also been used in a clinical trial involving 22 patients, unresponsive to an agressive treatment with conventional antiviral agents [41, 42].

The hepatitis B virus (HBV) represents the most widely diffuse chronic virus infection strictly related to the development of hepatocellular carcinoma. Several studies have been adressed to suppress HBV gene expression and cancer development; among them a paper by Moriya *et al.* [43], described the inhibition of HBV in the hepatocellular carcinoma mouse model. The series of antisense-treated mice showed the inhibition of the target gene in the liver. In addition, intraperitoneal (i.p.) injection of this aODN prevented pre-neoplastic lesions of the liver, without toxic side effects, after 8 weeks of treatment.

The control of hepatitis C virus (HCV) translation by antisense oligomers has also been explored in a recent paper by Vidalin *et al.* [44]. Inhibition assays were carried out in cell free translation systems (rabbit reticulocyte lysate and wheat germ extract) with natural and chemically modified oligomers (α-anomer and phosphorothioate) against a sequence within the HCV 5′ non-coding region. Both oligomers were found to be active in inhibiting HCV RNA translation and could provide the basis for the synthesis of specific antiviral agents to the full-length HCV genome also in HCV-infected cells.

Several studies also reported aODN antiviral activity to the Herpes simplex virus (HSV) *in vitro* [45]. Although *in vivo* attempts to control the HSV-1

have been documented by topical application of PS aODN (ISIS 1082) some years ago, the latest investigations are mainly focused on defining the *in vitro* toxicity profile of ISIS 1082 [46].

GENETIC DISEASES AND OTHERS

The potentiality of targeting genetic disorder alterations or inflammatory diseases has also been investigated.

Antisense oligonucleotides targeted to a mutation in the splice site of the human β-globin gene, causing a form of β thalassemia, have been used to reverse the aberrant splicing and gene function [47]. The restoration of β-globin by a PS oligoribonucleotide in mammalian cells, stably expressing the mutated β-globin gene, was dose-dependent and generated the correct transcript and protein which were stable for up to 72 h. Since aODNs should be active against splice sites in various pre-mRNAs and may be of clinical interest for the *in vivo* treatment of such disease, this report documents a novel extension of the antisense concept to restore, rather than inhibit, the function of a gene.

A recent paper by Nyce *et al.* [48] reported the *in vivo* use of aODN against adenosine, an endogenous purine that may be an important mediator of bronchial asthma. Asthmatic patients, with elevated concentrations of adenosine in the bronchoalveolar fluids, responded to adenosine challenge with marked airway obstruction. In this study the bronchial hyperresponsiveness has been reduced by aerosolized phosphorothioate aODNs, targeting the adenosine A receptor, in an allergic rabbit model of human asthma: aODNs reached the airway smooth muscle, specifically reducing adenosine A_1 receptor number and desensitizing the animals.

The potential value of local treatment of inflammatory diseases with aODN has been further confirmed by a paper dealing with experimental colitis in mice [49]. The NF-KB *p65* is a subunit of a family of transcription factors of lymphocytes and macrophages involved in the immune responses and inflammation via cytokines (IL-1, IL-56, TNF-α). The NF-KB *p65* is strongly activated in induced colitis, mimicking Crohn's disease in humans. The local administration of *p65* aODN downregulated its expression and abrogated histological signs and clinical symptoms of colitis in mice. Moreover, antisense effects were more pronounced than glucocorticoid treatment.

The ability to modulate the expression of genes that influence vascular cell proliferation could have an interesting impact on proliferative disorders of diverse origins, including atherosclerosis and restenosis following balloon angioplasty. Restenosis is due to the accumulation of vascular smooth muscle cells (VSMCs) and constitutes a major limiting factor for the complete recovery after angioplasty. Since c-*myb* and c-*myc* oncogenes seem to be implicated in the control of VSMC proliferation, antisense targeted to these oncogenes have shown an inhibition of VSMC proliferation *in vitro* and of restenosis in animal models [50–52].

PERSPECTIVES AND CONCLUSIONS

At present, therapeutic aODN are constituted by two classes of molecular compounds: oligonucleotides and expressed nucleotides. The first, as the basic antisense therapy has been widely described in this review; the second one, is now under investigation in several clinical trials. In fact, expressed nucleotides involve expression vectors (plasmid, adenoviral or retroviral) used for gene therapy, in which the vector is a promising delivery system for the transfer and the expression of antisense sequences in patient cells. The stable transfection of RNA antisense overcomes the ODN-mediated nonspecificity and provides a promising therapeutic strategy for gene expression modulation. The evaluation of such clinical genetic therapies are just commencing and their efficacy and safety will be evaluated in the future [53]. To date, the pharmacological, pharmacokinetic and pharmacodynamic features of antisense agents, in particular the phosphorothioate group, have provided a basis for achieving human clinical trials against diseases including AIDS and cancer. More detailed trials that employ the antisense include phase I and II studies on: HIV, CMV retinitis, Chron's disease, ulcerative colitis, rheumatoid arthritis, prevention of restenosis, non-Hodgkin's lymphoma, AML and CML bone marrow purging. These studies are under investigation and conclusive data should test the validity of the concept that antisense oligos can be therapeutic agents.

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Enhancement or inhibition of HIV-1 replication by intracellular expression of sense or antisense RNA targeted at different intermediates of reverse transcription

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Objectives: To construct retroviral vectors expressing sense or antisense RNA targeted at HIV reverse transcription intermediates, and to test the anti-HIV properties of these constructs in transduced T cells.

Design: Five double-copy retroviral vectors were constructed, in which the expression of the sense or antisense RNA corresponding to HIV minus- or plus-strand strong-stop DNA was driven by the human $tRNA^{met}$ promoter.

Method: The templates for the sense or antisense RNA were polymerase chain reaction-cloned from HIV pNL43 into a murine leukaemia virus-based vector and corresponding defective virions were packaged in PA317 cells. Human Jurkat T cells transduced with these vectors were challenged with HIV and monitored for viral RNA, viral DNA and p24 production for 23 weeks.

Results: Intracellular expression of HIV sense RU5 sequences (RNA complementary to minus-strand strong-stop DNA) enhanced HIV replication in T cells. Expression of HIV sense or antisense U3RU5 sequences (identical or complementary to plus-strand strong-stop DNA) conferred long-term inhibition of HIV replication, despite continuous presence of viral challenge in the transduced cell cultures.

Conclusion: Plus-strand strong-stop DNA as an intermediate in the early process of viral reverse transcription can be explored as an additional target for anti-HIV gene therapy.

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Keywords: Reverse transcription, retroviral vector, antisense RNA, gene therapy

Introduction

'Intracellular immunization' of progenitor or mature T cells and monocyte–macrophages against HIV-1 may provide a potent means by which to break the progressive cycles of viral infection and killing of CD4+ cells, which eventually result in CD4+ cell depletion and collapse of the immune system of HIV-infected individuals [1,2]. Both proteins and RNA can be used as the effector molecules for intracellular immunization. Expressing RNA rather than foreign proteins *in vivo* has the advantage of avoiding eradication of the transduced cells by the host immune responses, which otherwise might be triggered by the expression of foreign proteins. Strategies by which foreign RNA may be used to inhibit HIV replication can be divided into three groups: (i) antisense RNA or ribozymes targeted at a specific HIV mRNA, such as *tat* [3–5], *rev* [5], *gag* [4,6], *env* or other HIV mRNA [7,8]; (ii) sense RNA mimicking a secondary structure of HIV RNA that

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interacts with viral proteins vital for HIV replication, such as *rev*-responsive element decoy [9] and *trans*-activation response element decoy [10,11]; (iii) ribozymes targeted at some common untranslated, but essential sequence present in HIV transcripts, for example, the 5′ leading sequence and the poly A signal of HIV RNA, or the HIV packaging sequences [12–20]. All these approaches have been met with some success primarily at cell-culture level.

Most of the intracellular immunization strategies listed above affect steps in the HIV replication cycle that take place after the establishment of proviral DNA. We report here a novel strategy of intracellular immunization against HIV infection, targeted at the early steps of HIV reverse transcription (RTn). During retroviral RTn, two template switches occur. The first of these switches transfers the minus-strand strong-stop DNA from the 5′ end of one RNA molecule to the 3′ end of either a second, or the same RNA molecule. The second template switch is thought to transfer the plus-strand strong-stop DNA from the 5′ end to the 3′ end of the same molecule of the newly synthesized minus-strand viral DNA [21–26]. We have previously reported the detection of double-stranded strong-stop DNA in HIV acutely infected cells. Based on the terminal structure and the kinetics of synthesis of the double-stranded strong-stop DNA, we proposed that the second template switch involves the transient existence of a free plus-strand strong-stop DNA that is released from its template by displacement synthesis [26]. Thus, strong-stop DNAs as RTn intermediates in general and the transient-free plus-strand strong-stop DNA in particular, may serve as targets for antisense intervention. We have constructed a series of retroviral vectors in which the expression of different sense or antisense RNA targeted at RTn intermediates was driven by the human $tRNA^{met}$ promoter in a double-copy vector context [27], a design suitable for effective antisense RNA expression [28]. We show here that while intracellular expression of sense RU5 sequences (RNA complementary to minus-strand strong-stop DNA) enhanced HIV replication in T cells, expression of other anti-RTn RNAs led to long-term inhibition of HIV replication despite the continuous presence of viral challenge.

Materials and methods

Construction of retroviral vectors

HIV-1 DNA was polymerase chain reaction (PCR)-amplified from HIV infectious clone pNL43 (obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland, USA) using the strategy described previously [29]. All PCR products were cloned into pBluescript II KS– (Stratagene, La Jolla, California, USA), and their sequences were subsequently verified by DNA sequencing. The anti-RTn templates were inserted into the polylinker between the tRNA promoter and the RNA polymerase III transcription termination signal of a double-copy vector, pLSNP [28]. Unless stated otherwise, all the DNA cloning was performed according to established procedures [30].

Cell culture, transfection and transduction

The amphotropic packaging cell line PA317 [31] [American Type Culture Collection (ATCC)] and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL Life Technology, Gaithersberg, Maryland, USA) supplemented with 10% fetal calf serum (FCS; Commonwealth Serum Laboratory, Melbourne, Australia), 12 ng/ml penicillin, 160 ng/ml gentamycin and 2 mmol/l L-glutamine. Jurkat cells (ATCC) were grown in RPMI-1640 (Gibco) with 10% FCS, 12 ng/ml penicillin, 160 ng/ml gentamycin and 2 mmol/l L-glutamine. All the cells were free of *Mycoplasma* spp.

The retroviral vector DNAs were converted into defective virions by transfection into PA317 cells using the calcium phosphate precipitation procedure [30]. Forty-eight hours after transfection the conditioned medium containing the defective recombinant virus was collected by centrifugation at 4000 rpm for 10 min and stored at −70°C. Jurkat cells were transduced by incubation with the conditioned medium containing recombinant defective retrovirus in the presence of 4 μg/ml polybrene (Sigma, St Louis, Missouri, USA). Twenty-four hours after transduction, the cells were cultured in RPMI-1640 growth medium containing 400 μg/ml active geneticin (Gibco), with medium change twice weekly. Resistant cells that had been selected by growing in selection medium for more than 4 weeks were pooled for further experiments.

Cellular RNA and DNA analysis

Total RNA from parental or transduced Jurkat cells was isolated by the acidic guanidium thiocyanate phenol–chloroform method [32]. Cytoplasmic RNA was prepared by Nonidet-P40 lysis and sodium dodecyl sulphate (SDS)–proteinase K digestion [33]. To remove contaminating DNA, all the RNA samples were digested with DNase. Five micrograms of cellular RNA was incubated with 3 U RNase-free DNase (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany), 12 U RNasin (Promega, Madison, Wisconsin, USA), 100 mmol/l sodium acetate, 10 mmol/l dithiolthreitol, 5 mmol/l magnesium sulphate in a 100 μl reaction, at 37°C for 20 min. Under these conditions, 1 U RNase-free DNase had been shown in our experiments to digest 1 μg plasmid DNA to completion in 15 min at 37°C.

Anti-RTn RNA expression was detected by RNA-based PCR. Extracted RNA (5 μg) was reverse transcribed in the presence of the appropriate antisense primer for detecting sense RNA, or the appropriate sense primer for detecting antisense RNA (see below), using Moloney murine leukaemia virus reverse transcriptase (RT; Gibco) under conditions recommended by the manufacturer. One-quarter (5 μl) of the resultant cDNA was amplified after heating to 65°C for 10 min to inactivate the RT. A 50 μl reaction mixture composed of 5 μl cDNA, 50 mmol/l KCl, 10 mmol/l Tris-HCl pH 8.3, 1.5 mmol/l $MgCl_2$, 200 mmol/l of each dNTP, 1.25 U *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut, USA), and 50 pmol of each of the appropriate primers (see below), was initially heated at 94°C for 3 min and then incubated at 94°C and 64°C for 30 sec and 2 min, respectively, for 40 cycles in a DNA thermal cycler (Perkin-Elmer Cetus). Ten microlitre portions of each PCR product were eletrophoresed on 1.5% agarose gels, and DNA was visualized by ultraviolet fluorescence after staining with ethidium bromide. RNA samples did not give rise to detectable signals when PCR-amplified without prior reverse transcription reaction step.

Extrachromosomal and chromosomal DNA were purified separately by the Hirt method in the presence of proteinase K [34,35]. Total cellular DNA was prepared by proteinase K digestion. Briefly, 10^6 cells were pelleted by microcentrifuge and resuspended in 100 μl digestion buffer containing 50 mmol/l KCl, 10 mmol/l Tris-HCl pH 8.3, 1.5 mmol/l $MgCl_2$, 0.5% Triton X-100, and 200 μg/ml proteinase K. After incubation at 56°C for 1 h. the samples were heated at 95°C for 10 min to inactivate the enzyme. Five microlitres of each DNA preparation was used directly for PCR detection of HIV-1 *gag* DNA and cellular β-globin DNA in the presence of appropriate primers (see below). The thermal cycling programme was the same as that for RT-PCR. To prevent contamination, all tubes containing DNA samples were opened only inside a dedicated laminar flow hood. One-fifth (10 μl) of each PCR product was resolved by electrophoresing on a 1.5% agarose gel, and transferred to Hybond-N+ extra membrane (Amersham International, Amersham, Buckinghamshire, UK) using conditions recommended by the manufacturer. The membrane was prehybridized for 5 h and hybridized for 18 h at 50°C in 5× saline–sodium phosphate–EDTA solution (SSPE; 0.9 mol/l NaCl, 40 mmol/l sodium phosphate pH 7.7, 5 mmol/l EDTA), 5× Denhardt's solution, 0.5% SDS, 80 μg/ml denatured salmon sperm DNA and 2 pmol ^{32}P 5′-end-labelled *gag*-specific oligonucleotide probe (see below). After hybridization the membrane was washed in 6× SSPE, 0.1% SDS at 55°C for 2 h with three changes of washing solution and exposed to Kodak Bio Max film (Eastman Kodak, Rochester, New York, USA) at −70°C for 4–24 h. The HIV *gag* DNA standard was derived from a plasmid containing full-length HIV *gag*. The linearized plasmid DNA was diluted in Tris–EDTA buffer containing 1 μg/ml Jurkat cell DNA.

Thymidine uptake and cell-surface CD4 expression assays

The rate of cell proliferation of each transduced Jurkat cell culture was estimated by tritiated thymidine incorporation assay. Briefly, parental Jurkat cells or transduced cells were seeded into quadruplicate wells of a 96-well cell-culture plate at 2×10^5 cells in 200 μl RPMI growth medium per well. Twenty microlitres of [^{3}H]-thymidine (Amersham) at a concentration of 50 μCi/ml in RPMI growth medium was then added to each well to give a final concentration of 4.5 μCi/ml. After 23 h, the cells were harvested onto filter papers using a semiautomatic cell collector. The radioactivity of the filter was measured in a scintillation spectrometer. Expression of cell-surface CD4 was measured by flow cytometry [36].

HIV-1 infection and p24 assay

HIV-1_{NL43} virus stock was produced by transfecting HeLa-*tat* cells, a clonal HeLa derivative constitutively expressing HIV-1 *tat* (unpublished data), with DNA from a infectious provirus clone, pNL43. Briefly, the subconfluent HeLa-*tat* cells were transfected by calcium phosphate precipitation as mentioned previously. Forty-eight hours after transfection, the conditioned medium containing the virus was collected and stored at −70°C. A aliquot of above medium was thawed and the median tissue culture infective dose was determined by limiting dilution assay. For HIV challenge, transduced Jurkat cells were infected with HIV-1_{NL43} at a multiplicity of infection (MOI) of 0.01 for 24 h at 37°C. The infected cells were washed twice with serum-free RPMI medium and resuspended in growth medium. The culture supernatants were collected for p24 assay twice weekly at 1–4 weeks post-challenge, once weekly at 5–10 weeks post-challenge, and once every 2 weeks at 10–20 weeks post-challenge. HIV p24 concentration in the supernatant was determined by using a commercial HIV-1 p24 enzyme-linked immunosorbent assay kit (DuPont Medical Products, Boston, Massachusetts, USA).

Virus rescue

Transduced Jurkat cells that had been infected with HIV-1 and maintained for 20 weeks were mixed with parental Jurkat cells at a ratio of 1 : 2. After coculture for 3 days, another aliquot of parental Jurkat cells were added at a ratio of 1 : 1. Every week after starting the coculture an aliquot of 10^6 cells were taken for HIV DNA PCR.

Oligonucleotide primers and probes

All the oligonucleotides were synthesized by Bresatec

(Adelaide, South Australia). Their sequences are as follows:

HIV-1 *gag* primers and probe: Gag5$^{(+)}$, 5′ AGT ACC CTT CAG GAA CAA ATA GGA 3′; Gag6$^{(-)}$, 5′ CAT GCT GTC ATC ATT TCT TCT AGT GTC 3′; Gag probe$^{(-)}$, 5′ TCT GGG TTC GCA TTT TGG ACC ACA AAG 3′.

Human β-globin primers: β-*glo* 1$^{(+)}$, 5′ CAA CTT CAT CCA CGT TCA CC 3′; β-*glo* 2$^{(-)}$, 5′ GAA GAG CCA AGG ACA GGT AC 3′.

Primers for anti-RT RNA: R$^{1(+)}$, 5′ GGG TCT CTC TGG TTA GAC CAG ATC 3′; LTR2$^{(-)}$, 5′ AGA TCT CCT CTG GCT TTA CTT T 3′.

Human β-actin primers: β-*act* 1$^{(+)}$, 5′ CTC ACC ATG GAT GAT GAT ATC GCC GCG CTC 3′; β-*act* 2$^{(-)}$, 5′ CGC GCT CGG TGA GGA TCT TCA TGA GGT AGT 3′.

Sequences for HIV *gag* primers and probes, R$_1^{(+)}$ and LTR2$^{(-)}$ primers are according to Genbank ascession number M19921. Sequences for human β-actin primers are from Genbank ascession number X0035. Sequences for human β-globin primers are from Bauer *et al.* [37].

Results

Construction of anti-RTn expression retrovirus vectors and transduction of T cells

We constructed a series of retroviral vectors in which the expression of effector RNA (anti-RTn) was placed under the control of the human tRNA^{met} promoter in the context of a double-copy vector (Fig. 1). Anti-RTn 1 should express plus-sense RNA containing the RU5 sequence. This transcript, in theory, might hybridize with the minus-strand strong-stop DNA, the first intermediate of HIV RTn, and thus inhibit HIV replication. However, there is no evidence for the existence of a free single-strand minus strong-stop DNA as a target for hybridization. Anti-RTn 1 may therefore simply provide abundant initiating template to enhance the level of minus-strand strong-stop DNA synthesis and thus enhance RTn of the main body of viral RNA from the 3′ end [23]. Anti-RTn 2 was designed to express plus-sense RNA containing the full U3RU5 sequence of HIV. Anti-RTn 3 was a similar construct apart from the fact that the Sp1 sites and TATA box were deleted from the U3 region. This should result in the expression of plus-sense RNA containing a U3RU5 sequence with a defect in HIV promoter sequences. Anti-RTn 2 and 3 may compete with the free, transient single-stranded plus strong-stop DNA

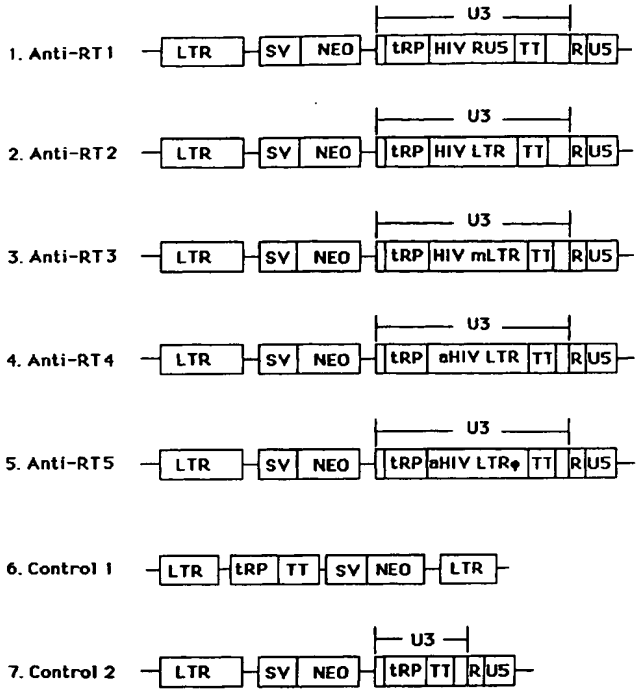

Fig. 1. Structure of anti-reverse transcription (anti-RTn) RNA expression vectors (not drawn to scale). HIV sequence numbers were based on HIV-1$_{NL43}$ (Genbank accession number M19921). The Moloney murine leukaemia virus 3′ LTR (LTR) was modified in vectors 1–5 and 7 as individually indicated. SV, Simian virus 40 early promoter; NEO, neomycin phosphotransferase gene; HIV LTR, LTR of HIV [nucleotides (nt) 1–684]; HIV RU5 (nt 454–684); HIV mLTR, HIV LTR with a deletion (nt 376–434) in the Sp1 sites and TATA box; aHIV LTR, template for antisense HIV LTR (nt 684–1); aHIV LTRφ, template for antisense HIV LTR plus HIV RNA packaging signal (nt 800–1); tRP, human tRNA^{met} promoter; TT, RNA polymerase III transcription termination signal.

[23,26], and thus interfere with the second template switch and inhibit HIV replication.

Anti-RTn 4 should express minus-sense RNA complementary to the plus-strand strong-stop DNA. Anti-RTn 5 had a similar structure to anti-RTn 4 except that the minus-sense RNA should also contain sequence complementary to the packaging sequence of HIV. Anti-RTn 4 and 5 RNAs may therefore anneal to the free plus-strand strong-stop DNA and interfere with the second template transfer, thus inhibiting virus replication. The control vectors had exactly the same structure as other vectors but without HIV sequences (Fig. 1).

The recombinant retrovirus DNA constructs were converted into defective virons which were used to transduce Jurkat cells. Each group of Jurkat transductants was separately selected in medium supplemented

with geneticin for 55–61 days until the number of cells reached similar levels in each group. Pooled cells corresponding to each construct were then used for further experiments without cell cloning. The Jurkat transductants are named after the corresponding vectors.

Anti-RTn RNA expression in Jurkat transductants

Intracellular expression of the anti-RTn RNA transcripts in Jurkat transductants was assessed by RT-PCR. Total cellular RNA was isolated from Jurkat transductants 8–10 weeks after selection in geneticin-containing medium as described in Materials and methods. Sense primer (R_1) was used to synthesize cDNA from any antisense RNA present, while the antisense primer (LTR2) was used to synthesized cDNA from the sense RNA (see Materials and methods). The cDNA was then amplified by PCR after inactivation of RT and addition of the second primer. Jurkat cells transduced by all the vectors expressed the expected sense or antisense RNA (Fig. 2A). The expression level of sense RNAs (anti-RTn 1, 2 and 3) was lower than that of antisense RNA (anti-RTn 4 and 5). The strand specificity of the RT-PCR procedure was confirmed by experiments using synthetic oligonucleotides complementary to the sense (or antisense) RNA as primers; these experiments demonstrated that RT-PCR with sense primers amplified only antisense targets and *vice versa* (data not shown).

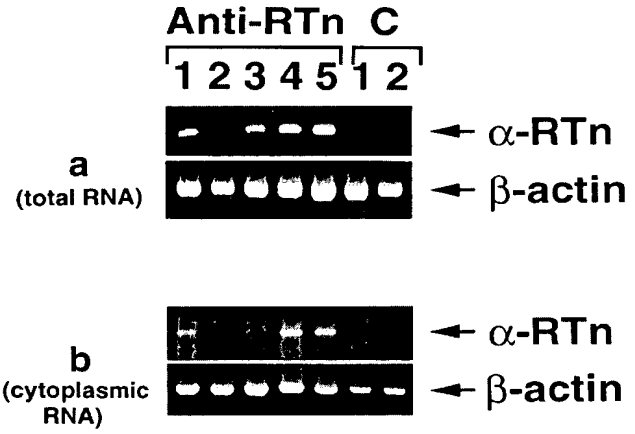

Fig. 2. Expression of anti-reverse transcription (anti-RTn) RNA in Jurkat cells transduced by retroviral vectors. Total cellular RNA or cytoplasmic RNA was extracted from Jurkat cells transduced by retroviral vectors anti-RTn 1–5 and control vectors, C1 and C2. Anti-RTn RNA expression was detected by reverse transcriptase-polymerase chain reaction (PCR) and ethidium bromide staining. (a) Total anti-RTn RNA in transduced cells 8 weeks after transduction. (b) Cytoplasmic anti-RTn RNA in transduced cells 9 weeks after transduction. A faint band was also discernible in track 3 (b) in the original photograph. α-RTn, anti-RTn RNA; β-actin, β-actin RNA.

To determine whether the anti-RTn RNA transcripts could be transported to the cytoplasm, we isolated cytoplasmic RNA from transduced cells and used RT-PCR to detect the anti-RTn RNAs (Fig. 2b). After allowing for a reduced yield of RNA in the cytoplasmic preparations, the expression pattern of anti-RTn RNA in the cytoplasm was similar to that in total cellular RNA. RT-PCR on nuclear RNA samples produced weaker signals than those of the corresponding cytoplasmic RNA samples (data not shown). This demonstrates that the majority of the anti-RTn RNAs could be transported to cytoplasm.

Anti-RTn RNA expression did not alter the rate of cell proliferation and surface CD4 expression

To determine the effect of anti-RTn RNA expression on the proliferation of T cells, [^{3}H]-thymidine uptake assays were performed. The rates of [^{3}H]-thymidine incorporation in Jurkat anti-RTn 1–5 cultures were similar to each other and to that of control cells and parental Jurkat cells (data not shown). Cell proliferation rates as indicated by the cell counts over a period of 4 weeks also did not show significant difference between the various groups of anti-RTn RNA-expressing cells and the control cells (data not shown). These results demonstrated that the expression of anti-RTn RNA did not have substantial effects on the proliferation of T cells.

The effect of anti-RTn RNA expression on cell-surface CD4 expression was evaluated by using flow cytometry. The overall levels of CD4 expression of the parental cells and each of the six groups of transduced cells were similar, although individual measurements of the cells in the same group varied, as indicated by the extent of SD in each group (data not shown). These results indicate that expression of anti-RTn RNA did not significantly alter the cell-surface CD4 level.

Long-term inhibition of HIV replication in T cells transduced by anti-RTn expression vectors

One of the possible approaches to gene therapy against HIV infection involves *ex vivo* transduction of autologous haematopoietic stem cells which are then engrafted back into HIV-infected patients. The transduced stem cells and their progeny cells, such as CD4+ T cells, monocytes and macrophages, may express anti-HIV RNA and may therefore resist HIV infection. It is clinically impractical and undesirable to clone the transduced stem cells before engrafting back into patients. Furthermore, individual clones derived from cells transduced with one particular construct may demonstrate variable degrees of resistance to HIV infection [9]. We therefore used the pooled transduced Jurkat cells derived from each construct, rather than homogeneous cloned cell lines, to test their ability to resist HIV infection. Results derived from analysing a whole population of transduced cells may minimize the bias associated with analysing clonal cells.

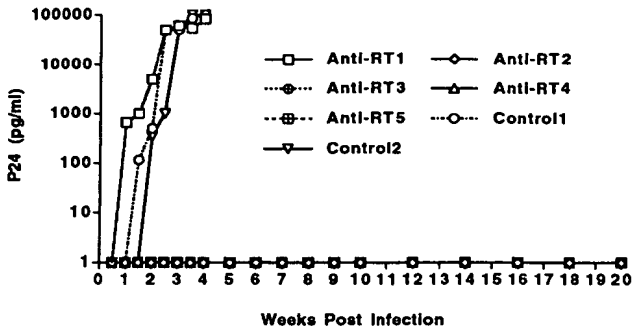

Fig. 3. Inhibition of HIV replication in transduced T cells expressing anti-reverse transcription (anti-RTn) RNA. Cell culture supernatants were collected at designated timepoints after HIV-1 challenge of Jurkat cells transduced by anti-RTn 1–5 and control 1 and 2. p24 concentration was determined by enzyme-linked immunosorbent assay. The results shown on the graph are the average value of two experiments. p24 levels exceeding 100 ng/ml (for Jurkat anti-RTn 1, Jurkat control 1, 2) were not further quantified.

Cultures of Jurkat cells transduced with each of the five constructs or the control constructs were challenged at MOI of 0.01 with HIV-1 virus stock prepared from the pNL43 infectious clone. NL43 was chosen for its ability to cause marked cytopathic effects and cell killing in parental Jurkat cells. To monitor the progress of infection, culture supernatants and cells were collected at designated timepoints after challenge for HIV p24 assay, HIV RNA RT-PCR and HIV DNA PCR.

After HIV-1 challenge, the two separate control cultures that had been transduced by the control vectors (without anti-RTn sequence) showed syncytium formation within 1 week, which reached a peak by 4 weeks. Most of the cells were killed by that time. Marked syncytium formation also appeared in Jurkat cells transduced by anti-RTn 1 within 1 week, which progressed more rapidly than in the control cells, and reached a peak on day 17. No cytopathic effects were observed in Jurkat anti-RTn 2, 3, 4 and 5 during 20 weeks of follow-up.

HIV replication as indicated by the appearance of p24 in the culture supernatants correlated well with the cytopathic effects (Fig. 3, Table 1). p24 appeared in the supernatant of the two control cultures 10 days after infection, and reached a peak at 4 weeks, whereas in Jurkat cells expressing sense RU5 RNA (Jurkat anti-RTn 1), p24 appeared earlier and reached a peak in a shorter time than in the control cells. In contrast, Jurkat anti-RTn 2, 3, 4 and 5 showed no detectable p24 secretion throughout the 20-week experiment. This suggested that no detectable amount of virus was released by Jurkat anti-RTn 2, 3, 4 and 5, following HIV challenge. To test whether the production of viral protein was also inhibited, we performed immunofluorescence assay 9 weeks after infection to detect p24 inside or on the surface of the cells. The results (date not shown) were in good agreement with that of culture supernatant p24 assay: HIV p24 was found only in Jurkat anti-RTn 1 and the control cells. No p24 immunofluorescence was detected in Jurkat anti-RTn 2, 3, 4 and 5 cells.

The levels of HIV proviral DNA produced following HIV challenge of Jurkat cells transduced with the different anti-RTn vectors or control vectors were monitored using semiquantitative PCR. To obtain more accurate quantification and avoid cross-contamination we have routinely used a simple and highly reproducible protocol to prepare cellular DNA samples from 1 million infected cells (see Materials and methods). Representative results of DNA PCR are shown in Fig. 4a (i, ii). After viral challenge, control cells and Jurkat anti-RTn 1 cells contained high levels of HIV proviral DNA at all the timepoints tested ($> 10^3$ copies in 5×10^4 cells; Table 1). In contrast, Jurkat anti-RTn 2, 3, 4, and 5 showed no detectable HIV DNA at most of the timepoints, and only low level of HIV DNA at some timepoints tested.

Three possible explanations may account for the absence or very low level of HIV DNA in these cultures. First, the cells may have been initially infected by HIV, but in the presence of anti-RTn sequences, the

Table 1. Long-term inhibition of HIV replication conferred by anti-reverse transcription (anti-RTn) RNA.

Vectors	HIV DNA* (weeks PC) 4	15	23	p24 ELISA	p24 IFA 9 weeks PC	HIV RNA 14 weeks PC	Virus rescue 20 weeks PC
Anti-RTn 1	$> 10^3$	$> 10^3$	$> 10^3$	+ (from 0.5 weeks)	+	+	+
Anti-RTn 2	0	5	< 1	–	–	–	+
Anti-RTn 3	0	0	0	–	–	–	+
Anti-RTn 4	0	0	0	–	–	–	+
Anti-RTn 5	0	0	0	–	–	–	+
Control 1	$> 10^3$	$> 10^3$	$> 10^3$	+ (from 1.5 weeks)	+	+	+
Control 2	$> 10^3$	$> 10^3$	$> 10^3$	+ (from 2 weeks)	+	+	+

*HIV proviral DNA copies in 5×10^4 cells. PC, Post-HIV challenge; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay.

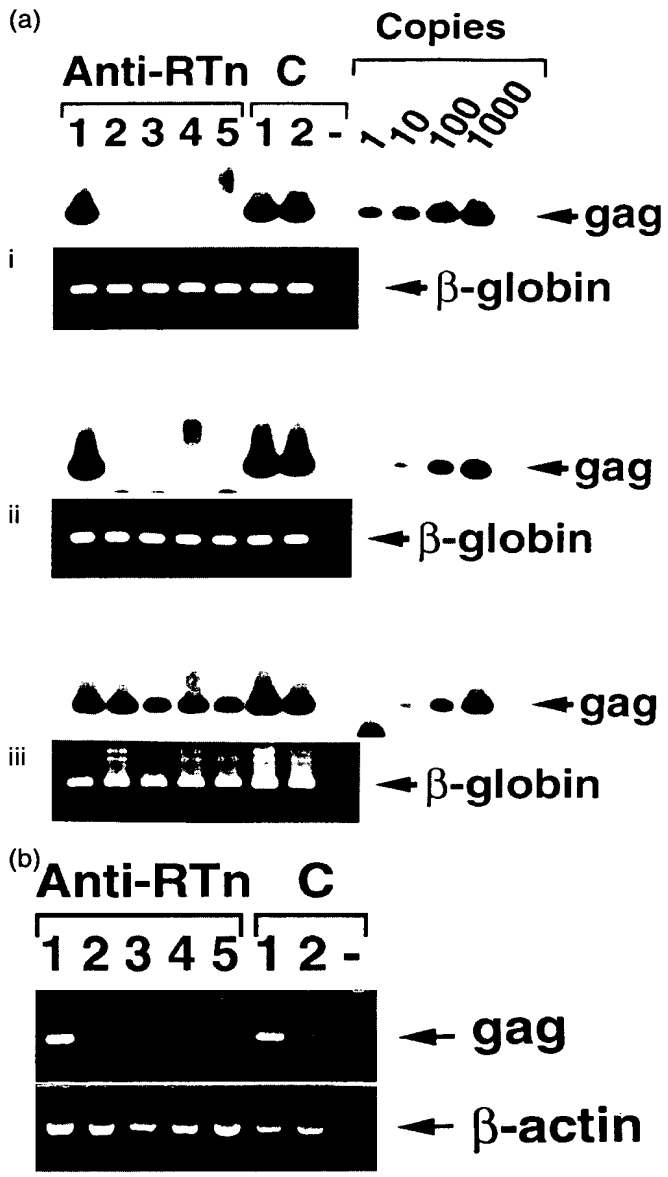

Fig. 4. Inhibition of HIV DNA and RNA synthesis by antisense RNA expression. (a) Total cellular DNA was prepared from transduced cells and subjected to polymerase chain reaction (PCR) using $Gag5^{(+)}$ and $Gag6^{(-)}$ primers. The PCR products were resolved on agarose gels and blotted onto nylon membranes and probed with HIV $Gag^{(-)}$ probe. Plasmid DNA containing the full-length HIV *gag* DNA was used as copy number standards. PCR on β-globin DNA was used to rule out the presence of PCR inhibitors and the variation in DNA input. (i) HIV *gag* DNA detected 18 weeks post-HIV challenge. (ii) HIV *gag* DNA detected 23 weeks post-HIV challenge. (iii) PCR detection of HIV *gag* DNA 1 week after virus rescue. (b) Total cellular RNA was isolated from the transduced cells 14 weeks after HIV-1 challenge. HIV-1 *gag* RNA was detected using reverse transcriptase (RT)-PCR. β-actin RNA was included as a control in RT-PCR using the same RNA samples.

virus was unable to complete RTn and any incomplete viral DNA initially produced was subsequently lost from the culture without becoming integrated. Second, proviral DNA was produced but was rendered defective by some of the anti-RTn RNA sequences during the second template transfer. Further viral replication would then not be able to take place. Third, the virus may have established productive infection in a minority of cells that had inadequate anti-RTn levels (as transduced cells were not cloned) but further viral replication and spread was continuously inhibited by the anti-RTn RNA expressed in the majority of transduced cells. To test these possibilities, we performed a virus rescue experiment. The rationale behind this experiment was that if the culture contained replication-competent virus that was continuously suppressed by the anti-RTn RNA, then following coculture with permissive cells, such as parental Jurkat cells, this virus should undergo a burst of replication. After coculture with parental Jurkat cells for 1 week, high levels ($> 10^3$ copies per 5×10^4 cells) of HIV DNA could be detected from Jurkat anti-RTn 2, 3, 4 and 5 (Fig. 4a iii). These results suggest that during the 20 weeks after initial HIV challenge, viable virus remained in the system, which was fully replication-competent. More importantly, it appeared that the expression of anti-RTn 2, 3, 4 and 5 continuously suppressed virus replication in the face of continuing HIV challenge.

To examine the effects of anti-RTn RNA expression on the transcription of HIV RNA after HIV challenge, we performed RT-PCR to detect HIV RNA. Total cellular RNA was isolated from Jurkat transductants 14 weeks after HIV infection. After digestion with RNase-free DNase to remove contaminating DNA, the RNA was subjected to RT-PCR to detect HIV *gag* RNA. As shown on Fig. 4b, Jurkat anti-RTn 1 and the control cells contained HIV RNA transcripts, whereas Jurkat anti-RTn 2, 3, 4 and 5 did not have detectable HIV *gag* RNA. These results demonstrated that the production of HIV RNA was inhibited in Jurkat cells expressing anti-RTn 2, 3, 4 and 5.

Discussion

Our experiments have demonstrated that intracellular expression of a number of different RNAs targeted at HIV plus-strand strong-stop DNA could confer long-term inhibition of HIV replication in otherwise fully permissive human T cells. In contrast, the expression of sense RNA complementary to the minus-strand strong-stop DNA actually enhanced HIV replication. It seems that the RU5 sequences expressed in the form of RNA were unable to anneal with the minus-strand strong-stop DNA in the process of first template transfer. Instead, the RU5 RNA molecules may have served

as initiating templates to enhance the synthesis of the minus-strand strong-stop DNA that would take part in the first template transfer and subsequently facilitate the synthesis of the major body of minus-strand DNA. This scenario would predict that U5 region of minus-strand strong-stop DNA be synthesized using the vector-expressed RU5 RNA as template [23]. The mechanism involved in the enhanced HIV replication in Jurkat anti-RTn 1 cells is currently under investigation.

A high level of inhibition of HIV replication was observed in cells transduced by anti-RTn 4, which expressed minus-sense RNA complementary to the plus-strand strong-stop DNA sequence. This minus-sense RNA may inhibit the second template switch by annealing with plus-strand strong-stop DNA. It could also bind to all the HIV mRNA that contained R and U5 sequence and therefore inhibit the translation of HIV proteins. A slightly less efficient inhibition of HIV production (as indicated by transient presence of low level HIV DNA, data not shown) was observed in cells transduced by anti-RTn 5, which has a similar structure to anti-RTn 4 except that the minus-sense RNA would also include a sequence complementary to part of the packaging sequence of HIV RNA. That part of the packaging sequence is not present in the plus-strand strong-stop DNA, but is present in the unspliced HIV RNA transcripts. The less potent inhibition by anti-RTn 5 may therefore suggest that anti-RTn 4 or 5 may have exerted their effects on HIV replication mainly by binding to the strong-stop DNA intermediates, although inhibition by annealing to HIV transcripts could not be ruled out.

Expression of anti-RTn 2 (sense RNA representing HIV U3RU5 sequence) and anti-RTn 3 (as above but with deletions in the Sp1 sites and TATA box) may inhibit HIV replication by competing with the transfer of functional plus-strand strong-stop DNA to newly synthesized minus-strand DNA. The sense U3RU5 RNA (anti-RTn 2) may also anneal to the minus-strand viral DNA and subsequently serve as a primer for plus-strand viral DNA extension, resulting in creation of a disabled double-stranded viral DNA with a DNA–RNA hybrid in the critical 5′ long terminal repeat region. If the deleted U3RU5 RNA (anti-RTn 3) annealed to the RU5 primer binding site sequence of minus-strand HIV DNA, the subsequent copying of the deleted U3 part into viral DNA will also generate a viral DNA without functional promoter. Such a structure has been proved replication deficient even in the presence of cellular activator [37]. Of the three sense constructs, the enhancing (anti-RTn 1) or inhibitory effects (anti-RTn 2 and 3) on HIV replication could be best interpreted if we assume that the second template switch involves a free, single-stranded (plus) strong-stop DNA whereas the first template switch does not [23,25,26]. As such, the first template switch may employ a structurally coordinated transfer mechanism where only the natural minus-strand strong-stop DNA (RU5) would be extended into the 3′ R region of the receiving RNA molecules. In this scenario, anti-RTn 2 and 3 can only play the inhibitory roles during the second template switch.

Our results indicate that the strong-stop DNA intermediates, which are involved in a different and earlier stage in the HIV replication cycle than the 'traditional' antisense targets such as *tat* or *rev* mRNA, can be efficiently targeted by intracellular immunization. A combination strategy targeting the early stage of HIV replication (anti-RTn) as well as the HIV expression stage (such as anti-*tat* or anti-*rev*) may enhance the efficiency of protection against HIV infection and may be useful for gene therapy against HIV infection and AIDS.

Acknowledgements

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T-cell-receptor signalling in inositol 1,4,5-trisphosphate receptor (IP_3R) type-1-deficient mice: is IP_3R type 1 essential for T-cell-receptor signalling?

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Stimulation of T-cells via the T-cell receptor (TCR) complex is accompanied by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Recently, it was reported that a stable transformant of the human T-cell line, Jurkat, expressing an antisense cDNA construct of inositol 1,4,5-trisphosphate receptor (IP_3R) type 1 (IP_3R1), failed to demonstrate increased $[Ca^{2+}]_i$ or interleukin-2 production after TCR stimulation and was also resistant to apoptotic stimuli. This cell line lacked IP_3R1 expression, but expressed the type-2 and -3 receptors, IP_3R2 and IP_3R3 respectively [Jayaraman, Ondriasova, Ondrias, Harnick and Marks (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 6007–6011, and Jayaraman and Marks (1997) Mol. Cell. Biol. **17**, 3005–3012]. The authors concluded that IP_3R1 is essential for TCR signalling and suggested that Ca^{2+} release via IP_3R1 is a critical mediator of apoptosis. To establish whether a loss of IP_3R1 function in T-cells occurred *in vivo* and *in vitro*, we investigated Ca^{2+} signalling after TCR stimulation and the properties of T-cells using IP_3R1-deficient ($IP_3R1-/-$) mice. As $IP_3R1-/-$ mice die at weaning, we transplanted bone marrow cells of $IP_3R1-/-$ mice into irradiated wild-type mice. Western blot analysis showed that the recipient IP_3R1-containing ($IP_3R1+/+$) lymphocytes were replaced by the donor $IP_3R1-/-$ lymphocytes after transplantation and that expression of IP_3R2 and IP_3R3 was unaltered. In contrast with the previous reports, T-cells lacking IP_3R1 were able to mobilize Ca^{2+} from intracellular Ca^{2+} stores after stimulation via the TCR. We observed no significant differences between $IP_3R1+/+$ and $IP_3R1-/-$ T-cells in terms of the number of thymocytes and splenocytes, the proportion of the T-cell phenotype, proliferative response to anti-CD3 monoclonal antibody (mAb) stimulation and cell viability. Therefore IP_3R1 is not essential for T-cell development and function.

INTRODUCTION

Activation of T-cells by a specific antigen is a fundamental event that is essential for the immune system to protect the host against pathogens. Stimulation of the T-cell receptor (TCR) complex is accompanied by the following sequential steps: activation of protein tyrosine kinases (PTKs), recruitment of the SH2 (phosphotyrosine-binding) domain containing phospholipase C-γ (PLCγ) and activation of PLCγ by tyrosine phosphorylation, resulting in phospholipid hydrolysis yielding diacylglycerol and D-*myo*-inositol 1,4,5-trisphosphate (IP_3). Both of these latter molecules function as intracellular second messengers: diacylglycerol activates protein kinase C (PKC) [1], which exerts its physiological function through phosphorylation of target proteins, whereas IP_3 binds to IP_3 receptors (IP_3Rs) and induces Ca^{2+} release into the cytoplasm from intracellular Ca^{2+} stores [2]. Subsequently, the depletion of Ca^{2+} stores activates Ca^{2+} influx through plasma-membrane channels, which causes a sustained increase in the free intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and, in turn, leads to the activation of various transcription factors and the expression of T-cell-specific functions [3–5].

T-cells express three types of IP_3Rs: IP_3R type-1 (IP_3R1), -2 (IP_3R2) and -3 (IP_3R3) [6–8]. It is uncertain whether each type of IP_3R has a specific function in TCR signalling. It was reported recently that a stable transformant of the human T-cell line, Jurkat, expressing an antisense cDNA construct of IP_3R1 failed to increase $[Ca^{2+}]_i$ or produce interleukin-2 after TCR stimulation [9] and was also resistant to apoptotic stimuli [8]. Because this cell line lacked IP_3R1 but expressed IP_3R2 and IP_3R3, the authors concluded that IP_3R1 was essential for TCR signalling and suggested that intracellular Ca^{2+} release via IP_3R1 is a critical mediator of apoptosis.

Recently, we have generated IP_3R1-deficient ($IP_3R1-/-$) mice using gene targeting. $IP_3R1-/-$ mice have a severely abnormal phenotype, which includes ataxia, epileptic seizures, small body size and death before weaning [10]. If IP_3R1 is essential for TCR signalling and apoptosis, we would expect $IP_3R1-/-$ mice to show abnormalities of T-cells and the immune system. For example, T-cells from these mice would lack both normal increases in $[Ca^{2+}]_i$ and cell proliferation after TCR stimulation and resistance to apoptosis should result in the failure of negative selection, thereby causing proportions of the

Abbreviations used: 7AAD, 7-aminoactinomycin D; BCR, B-cell antigen receptor; BM, bone marrow; $[Ca^{2+}]_i$, free intracellular $[Ca^{2+}]$; FITC, fluorescein isothiocyanate; IP_3, D-*myo*-inositol 1,4,5-trisphosphate; $IP_3R(n)$, IP_3 receptor (type n; $n = 1–3$); mAb, monoclonal antibody; PE, phycoerythrin; TCR, T-cell receptor.

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T-cell phenotype in the thymus to be abnormal. To test this hypothesis and to establish the result of a loss of IP_3R1 function in T-cells, we have produced $IP_3R1-/-$ mice with a pure genetic background of C57BL/6 and then transplanted the $IP_3R1-/-$ bone marrow (BM) cells into irradiated C57BL/6 mice to study the properties of $IP_3R1-/-$ T-cells *in vivo* and *in vitro* in terms of T-cell phenotype, proliferative response, Ca^{2+} mobilization after TCR stimulation and cell viability.

MATERIALS AND METHODS

Reagents and antibodies

Monoclonal antibodies (mAbs) 18A10, KM1083 and KM1082 against IP_3R1, IP_3R2 and IP_3R3 respectively were prepared as described previously [6,11]. Anti-CD3 mAb 2C11 (Cedarlane Lab. Ltd., Hornby, ON, Canada), phycoerythrin (PE)-labelled anti-Thy1.2 mAb (53-2.1; Phar Mingen, San Diego, CA, U.S.A.) and PE-labelled anti-CD4 mAb (GK1. 5, Phar Mingen) were purchased. Fluorescein isothiocyanate (FITC)-labelled anti-CD8 mAb (53.6.7) and FITC-labelled anti-B220 mAb (RA3/6B2) were obtained from American Type Culture Collection (Rockville, MD, U.S.A.).

$IP_3R1-/-$ mice and back-crossing

$IP_3R1-/-$ mice were generated using gene targeting [10]. In order to perform studies on the immune system, we produced (by back-crossing) $IP_3R1-/-$ mice with a pure C57BL/6 genetic background. Back-crossing was done using standard procedures [12]. The genetic background of the F2 generation of $IP_3R1-/-$ mice is composed of 129/Sv and C57BL/6 in the same ratio to each other. The 129/Sv background is derived from the J1 ES (embryonic stem) cell line, which was used to produce germ-line chimaeras in the process of generating $IP_3R1-/-$ mice. A male heterozygote F2 was mated with a female C57BL/6 mouse and its heterozygous male offspring were chosen for subsequent mating with female C57BL/6 mice; this process was repeated more than six times. On the first occasion that we obtained heterozygous female offspring during these repeats, another step was added in which the heterozygous female was mated with a C57BL/6 male to replace the Y-chromosome of F2 mice. After these crosses, in which the resultant background of a heterozygote was expected to be C57BL/6 in > 99% of cases, we then conducted double heterozygote crossing and obtained homozygotes ($IP_3R1-/-$) with a pure genetic background.

BM transplantation

Recipient 7-week-old male C57BL/6 mice received a lethal whole-body dose of radiation (1000 rad from a ^{137}Cs source) before the intravenous injection of 1×10^6 fresh, unseparated BM cells from either $IP_3R1+/+$ or $IP_3R1-/-$ male mice. All experiments using lymphocytes from the recipients were performed 7 to 11 weeks after transplantation.

Western blotting

Freshly isolated thymus and spleen were dispersed in RPMI 1640 medium [10% (v/v) fetal-calf serum] with a slide glass and passed through a 200-μm mesh nylon screen to obtain thymocytes and splenocytes respectively. Thymocytes (1×10^7 cells) and splenocytes (1×10^7 cells) were solubilized in 100 μl of lysis buffer [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA/1 mM 2-mercaptoethanol/10% (v/v) glycerol/1% (v/v) Triton X-100/0.1 mM PMSF/10 μM leupeptin/10 μM pepstatin A] on ice for 30 min. The insoluble fraction was removed by centrifugation at 15000 *g* for 15 min at 4 °C. Total cleared lysates were subjected to SDS/PAGE (5% gels), transferred to nitrocellulose and immunodetected with specific mAbs against each type of IP_3R.

$[Ca^{2+}]_i$ measurements in cell suspension

$[Ca^{2+}]_i$ was measured using Fura-2. Either thymocytes or splenocytes (1×10^7/ml) were loaded with 5 μM Fura-2 AM (Molecular Probes, Eugene, OR, U.S.A.) in RPMI 1640 medium [(2% (v/v) fetal-calf serum] containing 2 μM 2-mercaptoethanol. After incubation for 30 min at 37 °C, the cells were washed and resuspended in RPMI medium [10% (v/v) fetal-calf serum]. Before $[Ca^{2+}]_i$ measurement the cells were washed twice and then resuspended in a modified Krebs–Ringer solution [125 mM NaCl/5 mM KCl/1 mM $MgSO_4$/1 mM Na_3PO_4/1 mM $CaCl_2$/5.5 mM glucose/20 mM Hepes/KOH (pH 7.2)] to give a cell concentration of 4×10^6 cell/ml. The fluorescence of the stirred cell suspension was measured by using CAF-110 (Jasco, Tokyo, Japan) at 30 °C with $\lambda_{excitation}$ 340 nm and 380 nm and $\lambda_{emission}$ 510 nm. The $[Ca^{2+}]_i$ was calculated as described previously [13].

Immunofluorescence analysis

mAbs used were PE-labelled anti-Thy1.2, PE-labelled anti-CD4, FITC-labelled anti-CD8 and FITC-labelled anti-B220. After the staining, the cells were suspended in buffer containing 1 μg/ml 7-aminoactinomycin D (7AAD, Sigma) to exclude dead cells from the analysis. Stained cells were analysed on a FACscan (Becton Dickinson, Mountain View, CA, U.S.A.). Only cells in the lymphocyte gate (as defined according to side and forward scatters) were analysed.

Cell culture and proliferation assay

Cells were cultured in RPMI 1640 medium [10% (v/v) fetal-calf serum] supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 2 μM 2-mercaptoethanol. For proliferation assays, cells were cultured at 2×10^5 cells/well in a 96-well flat-bottom plate with concanavalin A (5 μg/ml) or various concentrations of immobilized anti-CD3 mAb. The cells were pulse-labelled with [^{3}H]thymidine (0.2 μCi/well) during the last 8 h of a 72 h culture period and [^{3}H]thymidine incorporated was measured by a MATRIX 96 Direct β-Counter (Hewlett–Packard, Boise, ID, U.S.A.). All assays were done in triplicate.

RESULTS AND DISCUSSION

$IP_3R1-/-$ mice with a pure C57BL/6 genetic background showed the same phenotype as that of F2 mice, which included ataxia, epileptic seizures, a small body size and death before weaning [10]. To investigate a loss of function of IP_3R1 and to clarify the significance of IP_3R1 in T-cells, we needed to eliminate any effects resulting from undernourishment and stress by the severe phenotypic changes on the immune system. Therefore, we transplanted the BM cells of either $IP_3R1+/+$ (control) or $IP_3R1-/-$ mice into irradiated wild-type mice and studied development and function of T-cells from the recipient mice. Both recipient mice transplanted with the $IP_3R1+/+$ BM cells and the $IP_3R1-/-$ ones grew normally and no differences in the phenotypic appearance was observed.

To confirm whether the donor $IP_3R1-/-$ BM cells replaced the original $IP_3R1+/+$ BM cells, the recipient thymocytes and splenocytes were subjected to Western blot analysis with the

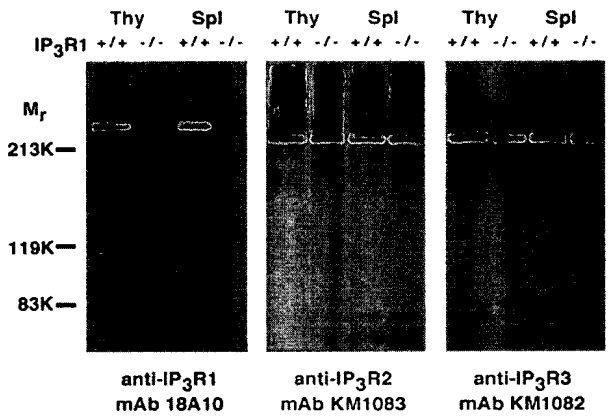

Figure 1 Western blot analysis of thymocytes and splenocytes lacking IP_3R1

Western blot analysis of total cell lysates of thymocytes (thy) and splenocytes (spl) (1×10^6 cells/lane) from the recipient mice transplanted with $IP_3R1+/+$ or $IP_3R1-/-$ BM cells with specific mAbs against IP_3R1, IP_3R2 and IP_3R3. Molecular mass markers are indicated on the left.

Table 1 Immunofluorescence analysis of thymocytes and splenocytes

Results were expressed as mean percentages ± S.D. ($n = 5$).

Property	Genetic phenotype IP3R1 +/+	IP3R1 −/−
Cell number		
Thymocyte $\times 10^7$	9.40 ± 3.24	10.3 ± 3.46
Splenocyte $\times 10^7$	11.5 ± 1.46	9.62 ± 2.37
Thymocyte phenotype		
DN %	2.42 ± 0.721	2.39 ± 0.954
DP %	85.4 ± 3.98	83.6 ± 2.70
SP(CD4+) %	8.97 ± 3.35	10.3 ± 1.05
SP(CD8+) %	3.18 ± 1.04	3.72 ± 1.19
Splenocyte phenotype		
B220+ (B-cell) %	63.2 ± 4.02	64.1 ± 5.92
Thy1+ (T-cell) %	27.8 ± 6.12	22.3 ± 5.62
Splenic T-cell phenotype		
CD4+CD8− %	22.0 ± 3.53	18.1 ± 2.59
CD4−CD8+ %	8.70 ± 1.25	7.04 ± 2.21
Ratio (CD4+/CD8+)	2.53	2.57

specific mAb against IP_3R1, 18A10 (Figure 1). The IP_3R1 was not detectable in thymocytes of the recipient mice; however, a faint but negligible band of IP_3R1 was detectable in splenocytes, which was estimated to be 0.88 ± 0.62 % (mean ± S.D., $n = 3$) of the control splenocytes by densitometric analysis using NIH Image 1.58 (National Institutes of Health, Bethesda, MD, U.S.A.), indicating that the recipient original $IP_3R1+/+$ BM cells were replaced by the donor $IP_3R1-/-$ BM cells after transplantation. This residual, but negligible, expression of IP_3R1 was possibly derived from the residual recipient IP3R1+/+ lymphocytes or from contamination of other tissues. The expression of IP_3R2 and IP_3R3 was also analysed by Western blotting with the specific mAbs KM1083 and KM1082 for IP_3R2 and IP_3R3 respectively (Figure 1). In contrast, the expression levels of both IP_3R2 and IP_3R3 were unaltered, suggesting that no significant compensation for loss of IP_3R1 by IP_3R2 and IP_3R3 occurred.

As demonstrated in the Jurkat cells lacking IP_3R1, if the IP_3R1 is essential for TCR signalling and if the IP_3R1 deficiency results in resistance to apoptosis [8,9], $IP_3R1-/-$ T-cells would show an abnormal phenotype, e.g., neither $[Ca^{2+}]_i$ increases nor cell proliferation would be observed after TCR stimulation. In addition, apoptosis resistance would result in loss of negative selection, thereby causing proportions of the T-cell phenotype in the thymus to be abnormal. We investigated the properties of T-cells *in vivo* by immunofluorescence analysis. The cell number of the $IP_3R1-/-$ thymocytes and splenocytes were almost the same as those of the control (Table 1). Quantification of the CD4+ and CD8+ populations using immunofluorescence analysis revealed that, in both thymocytes and splenocytes, these T-cell populations were unaltered, suggesting that the $IP_3R1-/-$ T-cells differentiate normally and that IP_3R1 deficiency does not result in the failure of apoptosis during negative selection (Table 1).

We then investigated the function of IP_3R1 in primary T-cells. We examined the proliferative response to TCR stimulation. Table 2 summarizes [^{3}H]thymidine incorporation upon TCR stimuli by anti-CD3 mAb. The IP_3R1-deficient T-cells in both thymocytes and splenocytes showed normal proliferative responses to stimulation *in vitro*, indicating that the IP_3R1 is not essential for proliferation of T-cells via TCRs. The cell viability of primary cells was also examined. The cells from $IP_3R1-/-$ recipient mice were cultured for 24 h in culture medium, and then the percentage of viable cells was determined on a FACScan after staining with 7AAD. There was no difference between the cell viability of IP3R1−/− T-cells and that of the control (results not shown). This result suggests that IP_3R1 is not an essential mediator of the apoptotic signal in primary T-cells.

Figure 2(A) shows typical profiles of $[Ca^{2+}]_i$ increases after TCR stimulation with the anti-CD3 mAb 2C11. In contrast with the Jurkat cells lacking IP_3R1 [9], both thymocytes and splenocytes lacking IP_3R1 were capable of increasing $[Ca^{2+}]_i$ after stimulation with anti-CD3 mAb in a similar manner to the control cells (Figure 2A). In Ca^{2+}-free solution, the initial rapid $[Ca^{2+}]_i$ increase, but not the subsequent sustained $[Ca^{2+}]_i$ influx, was observed (results not shown), confirming the previous observations that the initial $[Ca^{2+}]_i$ increase is due to Ca^{2+} release from the intracellular store and the subsequent sustained $[Ca^{2+}]_i$ increase is due to Ca^{2+} influx from extracellular medium. The maximal $[Ca^{2+}]_i$ increases ($n = 5$) are summarized in Figure 2(B) ($n = 5$). There were no differences between $IP_3R1+/+$ and $IP_3R1-/-$, suggesting that a loss of IP_3R1 function did not affect Ca^{2+} mobilization after TCR stimulation. We also examined $[Ca^{2+}]_i$ increases after TCR stimulation in the thymocytes and splenocytes from 17-day-old $IP_3R1-/-$ mice (C57BL/6). Stimulation of the $IP_3R1-/-$ thymocytes and splenocytes with anti-CD3 mAb also resulted in the same responses (results not shown) and immunofluorescence analysis also revealed that, in both thymocytes and splenocytes, these T-cells differentiate normally (results not shown), confirming that our results obtained using the transplanted mice reflect a loss of IP_3R1 function in T-cells. These results indicate that IP_3R1 is not essential for either Ca^{2+} mobilization after TCR stimulation or cell proliferation and thus the remaining types of IP_3R, IP_3R2 and IP_3R3, are sufficient for Ca^{2+} mobilization and cell proliferation.

The discrepancy between the results reported for Jurkat cells

Table 2 Proliferative response of thymocytes and splenocytes to anti-CD3 mAb

Thymocytes and splenocytes were examined for proliferative response to the indicated stimuli. Results were expressed as mean c.p.m ± S.D. of triplicate wells. Representative results of three independent experiments are shown.

Thymocytes

		Immobilized anti-CD3			
Stimulant...	None	0.4 μg/ml	2.0 μg/ml	10 μg/ml	Concanavalin A (5 μg/ml)
IP3R1 +/+	27 ± 7	1680 ± 610	4260 ± 200	8620 ± 1650	12300 ± 2100
IP3R1 −/−	59 ± 17	1240 ± 670	5260 ± 600	7420 ± 1480	10900 ± 1800

Splenocytes

		Immobilized anti-CD3			
Stimulant...	None	0.016 μg/ml	0.08 μg/ml	0.4 μg/ml	Concanavalin A (5 μg/ml)
IP3R1 +/+	175 ± 37	26000 ± 2000	32800 ± 1800	34000 ± 800	41400 ± 2300
IP3R1 −/−	348 ± 154	26200 ± 3700	31700 ± 1100	34300 ± 1600	38400 ± 3300

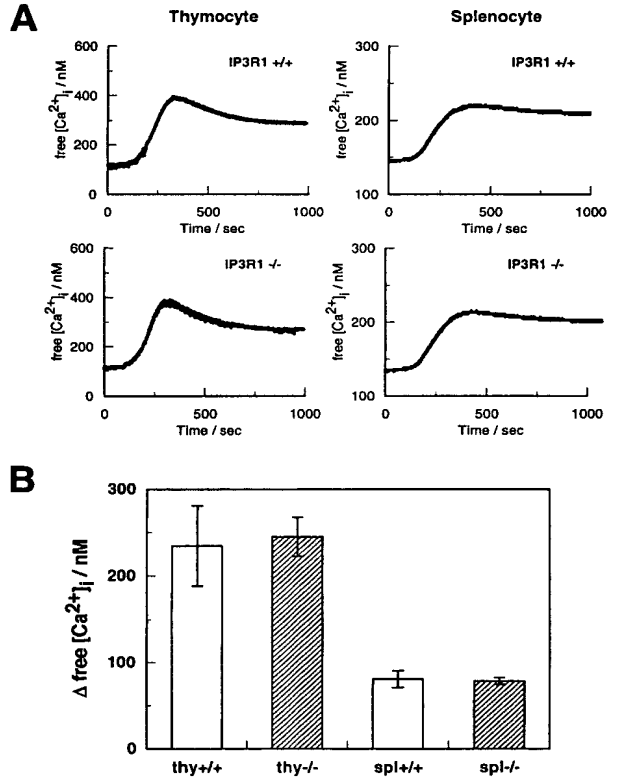

Figure 2 TCR stimulation with anti-CD3 mAb-induced $[Ca^{2+}]_i$ increase in the T-cells lacking IP_3R1

(**A**) The IP_3R1 +/+ and IP_3R1 −/− thymocytes and splenocytes were stimulated with anti-CD3 mAb 2C11 (2.5 μg/ml) at $t = 100$ s. Anti-CD3 mAb induced an initial $[Ca^{2+}]_i$ increase, followed by a sustained $[Ca^{2+}]_i$ increase, in all cases. (**B**) The $[Ca^{2+}]_i$ responses on TCR stimulation with anti-CD3 mAb are summarized. Error bars represent means ± S.D. ($n = 5$). thy, thymocytes; spl, splenocytes.

lacking IP_3R1 [8,9] and this study using IP_3R1 −/− T-cells might be due to the different cell-types used. In this case, the IP_3R1 might be the dominant type of IP_3R in the Jurkat cells and the expression of IP_3R2 and IP_3R3 might be too low to function as the Ca^{2+}-mobilizing channels in TCR signalling. Alternatively, the discrepancy may ensue from the different preparation of IP_3R1-deficient T-cells. In this study, we produced T-cells lacking IP_3R1 using gene targeting and transplantation, in which the IP_3R1 gene was specifically disrupted and this disruption did not affect the expression of IP_3R2 and IP_3R3. Therefore we could evaluate the significance of IP_3R1 in TCR signalling *in vivo* and *in vitro*. In contrast, Jurkat cells lacking IP_3R1 were established by the stable expression of an antisense cDNA construct of IP_3R1 to prevent the efficient translation of IP_3R1 mRNA. Using this strategy, the authors selected the 5′-region of 2.9 kb (nt −247 to −2592) in the human IP_3R1 cDNA for the antisense construct, which has high homology with the IP_3R2 and IP_3R3 (60–75%) [8,14–16]. Therefore it is possible that the antisense construct of IP_3R1 inhibited not only IP_3R1 expression, but also IP_3R2 and IP_3R3 expression, resulting in the observed failure to mobilize Ca^{2+} from intracellular Ca^{2+} stores after TCR stimulation. In fact the expression of IP_3R2 and IP_3R3 in the IP_3R1-deficient Jurkat cells was detectable, but reduced [8]. If this is the case, it is possible to conclude that only the IP_3R-1, -2 and -3 triple-deficient T-cells result in loss of Ca^{2+} mobilization after TCR activation, and that Ca^{2+} released via not only IP_3R1 but also IP_3R2 and IP_3R3 might be essential for TCR signalling and a critical mediator of apoptosis. Recently, Sugawara et al. [17] demonstrated that DT40 cells (a chick B-cell line) in which a single type of IP_3R has been deleted by gene targeting mobilize Ca^{2+} in response to B-cell antigen receptor (BCR) stimulation, whereas this Ca^{2+} mobilization is abolished in B-cells lacking all three types of IP_3R and the BCR-induced apoptosis is inhibited only in triple-deficient cells. We also observed that the IP_3R1 −/− splenocytes showed normal Ca^{2+} mobilization, normal cell proliferation after BCR stimulation with the anti-(mouse IgM) and normal cell viability (results not shown). Therefore the observations of Sugawara et al. and ourselves strongly suggest that not only IP_3R1 but also IP_3R2 and IP_3R3 are essential for BCR or TCR signalling and apoptosis. A third possibility for the discrepancy is that the antisense cDNA construct of IP_3R1 could

cause additional side effects on the expression of other proteins involved in TCR signalling.

In conclusion, we have analysed a loss of function of the IP_3R1 in T-cells in terms of T-cell development, Ca^{2+} mobilization and cell proliferation after TCR stimulation and cell viability. In clear contrast with the previously reported observations in Jurkat cells lacking IP_3R1 [8,9], we have observed no significant differences in several experiments between $IP_3R1+/+$ and $IP_3R1-/-$ T-cells, i.e. $IP_3R1-/-$ T-cells were capable of mobilizing Ca^{2+} after TCR stimulation and showed normal cell differentiation, proliferation and viability. These results indicate that IP_3R1 is not the sole receptor responsible for either Ca^{2+} mobilization after TCR activation or for cell proliferation, and that the remaining IP_3R2 and IP_3R3 are sufficient for T-cell development and function even in the absence of IP_3R1.

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Efficacy of p120 Antisense–Mediated Therapy for Pancreatic Cancer

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p120 Antisense oligodeoxynucleotides were used to determine whether they inhibited cell growth of MIA PaCa-2, a highly tumorigenic human pancreatic carcinoma cell line. Growth inhibition assays were determined in vitro by the ability of these oligomers to inhibit DNA synthesis and cell growth. For in vivo studies, nude mice were injected with cells and palpable tumors were found in 16 of 20 animals by day 14. Sixteen animals (8 in each group) were then treated daily (25 mg/kg intraperitoneally) for up to 40 days with nonsense control oligomers or p120 antisense oligomers. p120 Antisense oligomers inhibited the in vitro proliferation of MIA PaCa-2 cells in a dose-dependent manner, and optimal growth inhibition of greater than 90% was achieved at an antisense oligomer concentration of 100 μmol/L. The tumor volume was calculated for antisense- and nonsense-treated animals. Fifteen days after the beginning of treatment, control animals had a significantly greater ($P = 0.0035$) tumor volume (425 ± 244 mm^3 above baseline) as compared to p120 antisense–treated animals (166 ± 116 mm^3). Seven of the eight control animals formed tumors that had a volume greater than 1200 mm^3 45 days after treatment was begun, whereas only three of eight p120 antisense–treated animals had tumors that were this large. Two of the latter three animals had relatively large, palpable tumors (>150 mm^3) prior to treatment. Twenty days after treatment was stopped (day 60), all animals had tumors larger than 1200 mm^3. p120 Antisense oligomers were effective for inhibiting in vitro growth of the pancreatic cancer cell line MIA PaCa-2. In preliminary studies, p120 antisense oligomers appeared to inhibit the rate of growth in nude mice; however, no cures were achieved. The most effective response was seen in animals with initial low tumor burden. (J GASTROINTEST SURG 1997;1:454-460.)

Carcinoma of the exocrine pancreas is the fifth leading cause of cancer deaths in Western society and one of the leading causes of cancer deaths world wide.[1-3] There has been little improvement in patient survival in this century; the 5-year survival rate of patients with this disease is less than 5%, and the only cure is surgical resection.[1,2] Although surgery is considered the only curative modality, less than 10% of the patients have a localized tumor and thus are candidates for surgical resection. Despite advances in chemotherapy, radiation therapy, and surgical procedures, the overall impact of conventional therapeutic modalities has been minor in the management of pancreatic cancer.

Newer approaches now being considered are the use of neoadjuvant therapy and surgery combined with biologic markers to better predict and specify the behavior of these tumors. Other approaches include identifying genetic differences between normal malignant tissues with the intention of using gene therapy to replace tumor supressor genes or to inhibit the expression of oncogenes or other growth regulatory molecules expressed by tumor cells. A number of such molecules exist in pancreatic cancer including activated oncogenes and the overexpression of growth regulatory molecules.[4-6] The selective inhibition of growth regulatory molecules could potentially reverse the malignant phenotype, inhibit growth, and/or induce cell death. One approach for the selective inhibition of gene expression has been the use of antisense oligodeoxynucleotides.[7-12] Antisense oligodeoxynucleotides are short nucleotide sequences that are com-

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plementary to messenger RNA and to one strand of DNA. These types of antisense molecules can inhibit specific gene expression by several different mechanisms including transcriptional arrest, inhibiting initiation or elongation of transcription, inhibiting splicing, and promoting messenger RNA degradation via activation of RNAse H.

One potential growth regulatory molecule that appears to be overexpressed in tumor tissues and represents a downstream target of oncogenic changes is the p120 protein.[13-15] p120 Is a proliferation-associated nucleolar protein that is temporally expressed at a specific time during mid-G_1.[16] Studies have indicated that p120 expression is required for ribosomal biogenesis punitively through its role as an RNA methyltransferase.[17] Antisense-mediated specific inhibition of p120 expression has been shown to inhibit the growth of breast cancer cell lines[13] and to block G_1 to S-phase transition in mitogen-stimulated lymphocytes.[18] The present study was undertaken to determine the efficacy of p120 antisense oligodeoxynucleotide therapy for inhibiting the growth of pancreatic cancer cells. For this initial study, a highly tumorigenic pancreatic carcinoma cell line, MIA PaCa-2, was selected. MIA PaCa-2 cells have multiple genetic mutations including *ras*, p53, and the tumor growth factor-β type II receptor gene.[19,20] This cell line is growth factor independent, grows well in soft agar, and rapidly forms tumors in nude mice.[20]

MATERIAL AND METHODS

The MIA PaCa-2 pancreatic carcinoma cell line was purchased from American Type Culture Collection (Rockville, Md.), and cells were cultured using standard conditions as previously described.[20]

Synthesis of Oligomers

Two separate 15 mer antisense oligodeoxynucleotides were synthesized. One oligonucleotide was prepared as a phosphodiester (p120 antisense-sj). This oligomer was directed against a region of the gene that overlaps the eleventh splice junction. The second oligonucleotide was directed against the AUG-transcriptional start site of the p120 gene (p120 antisense-aug) and was prepared as a phosphorothioate. The nonsense oligomer for in vitro studies was a 15 mer phosphodiester oligodeoxynucleotide with the same nucleotides in the p120 antisense-sj but in a random "nonsense order." The nonsense control for the in vivo studies was a phosphorothioate oligodeoxynucleotide (Isis-1082) that has been previously described.[21]

Proliferation Assays

Cell proliferation was measured by the ability of ^{3}H-thymidine to be incorporated into DNA as previously described.[15] Cells were plated in six replicates in 96-well plates at 5×10^3 cells per 100 μl. The next day cells were left untreated or antisense or nonsense oligomers were added and the cells were cultured for an additional 24 hours. At that time an additional 50 μl of medium containing 1 mCi of ^{3}H-thymidine was added to each well for an additional 4 hours incubation, and the cells were then harvested and thymidine incorporation determined by liquid scintillation counting. Growth was also determined by the ability of viable cells to metabolize 3-[4,5-dimethylthiazol-2-yl]-2,5-diphentetrazolium bromide (MTT) metabolic labeling assay.[22] For this assay, cells were plated as above and treated with antisense or nonsense oligomers, and MTT assays were performed daily over a 7-day period. MTT was added at a final concentration of 0.5 mmol/ml. After 2 hours' incubation at 37° C, the medium was removed and cells were lysed in dimethyl sulfoxide. Color intensity was measured with a spectrophotometer at 590 nm. Results in the untreated group of cells were not significantly different from those in nonsense-treated cells and are therefore not shown.

Western Blot Analysis

Treated and control cells were washed and resuspended in Laemmli buffer at a concentration of 2×10^7 cells/ml. An equivalent of 10^5 cells were subjected to electrophoresis in each lane of a 7.5% polyacrylamide gel containing 0.1% sodium dodecylsulfate. The p120 protein was detected following Western transfer by immunoalkaline phosphatase as previously described.[16]

Therapeutic Effects of p120 Antisense

The p120 antisense directed against the AUG transcriptional start site (p120 antisense-aug) was used for all in vivo testing. This oligomer was chosen since it was prepared as a phosphorothioate and may therefore have a longer in vivo half-life than phosphodiester oligomers.[21] For these assays, MIA PaCa-2 cells were inoculated (2×10^6 cells per mouse) into the flank of NCr nu/nu nude mice. Animals were monitored and tumors were apparent by day 14, at which time treatment was begun. Animals were given intraperitoneal injections daily at a concentration of 25 mg/kg. This concentration of phosphorothioate oligodeoxynucleotides was chosen as an optimal nontoxic dose based on previous experiments using ro-

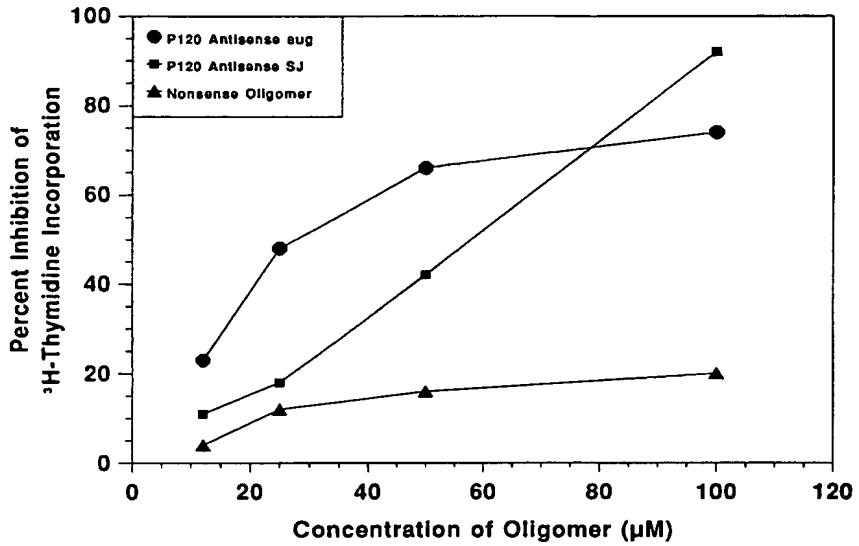

Fig. 1. The effect of p120 antisense oligomers on DNA synthesis was determined by ^{3}H-thymidine incorporation assay. The percentage of inhibition represents the ^{3}H-thymidine counts per minute of treated cells divided by the ^{3}H-thymidine counts per minute of untreated control cells × 100. The results were plotted as the mean of six replicates and the standard deviation was less than 13% for each mean.

dent models.[23] Animals were treated with oligomers for up to 40 days. The tumor volume was estimated following measurements of the length and width using the method of Shin.[24] A statistical comparison was made by means of Student's *t* test between the control and treated groups. Animals were killed after they developed tumors larger than 1200 mm^3.

RESULTS

p120 Antisense-Mediated Inhibition of Cell Proliferation and p120 Expression

The treatment of MIA PaCa-2 cells with p120 antisense oligodeoxynucleotides inhibited the in vitro growth of these cells. Cell growth was first measured by determining the effect of various concentrations of two different p120 antisense molecules and a p120 nonsense control molecule on DNA synthesis as measured by ^{3}H-thymidine incorporation. Both p120 antisense molecules inhibited DNA synthesis in a dose-dependent manner (Fig. 1). However, the kinetics of the inhibition differed between the two antisense molecules. The p120 nonsense control oligodeoxynucleotide showed a slight inhibition of DNA synthesis (<20% at 100 μmol/L). The p120 antisense-aug oligomer showed a greater growth inhibition at low concentrations (almost 50% at 25 μmol/L) than the p120 antisense-sj oligomer (17% at 25 μmol/L). There was a plateau effect on growth inhibition by the p120 antisense-aug oligomer at a concentration between 50 and 100 μmol/L. The p120 antisense-sj oligomer did not show a plateau at the concentrations of oligomer used and reached a maximum inhibition of DNA synthesis of greater than 90% at a concentration of 100 μmol/L. A single 100 μmol/L dose of either antisense oligomer completely inhibited any increase in cell numbers in MIA PaCa-2 cells cultured over a 7-day period (Fig. 2). The effect of treatment with p120 antisense-sj was analyzed for its ability to inhibit p120 protein expression. When compared to the nonsense control (Fig. 3, lanes 2, 4, and 6), the p120 antisense oligomer specifically inhibited p120 expression in a dose-dependent manner (Fig. 3, lanes 1, 3, and 5).

p120 Antisense-Mediated Inhibition of Pancreatic Tumor Growth in Nude Mice

The potential efficacy of p120 antisense oligomers to inhibit tumor growth in vivo was examined using a nude mouse model. Sixteen mice bearing palpable MIA PaCa-2 tumors (14 days after they were injected with cells) were randomly placed into two separate groups and treated for 40 days with either antisense (p120 antisense-aug) or nonsense control oligomers. The tumor volume for each treatment group is shown for days 1, 15, and 45 after treatment was initiated

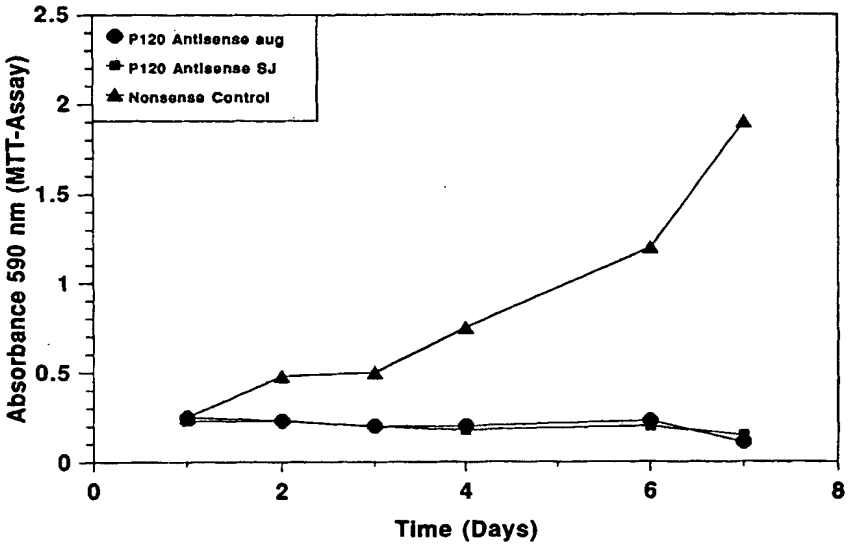

Fig. 2. The effect of a single treatment of the p120 antisense or nonsense oligonucleotide on the growth of MIA PaCa-2 cells was determined over a 7-day period. Growth was determined by a metabolic MTT assay using six replicate measurements. The standard deviation was less than 0.5 for each mean. Cells were treated on day 1 with a 100 μmol/L concentration of antisense or nonsense oligonucleotide. Metabolic labeling was recorded over a 7-day period.

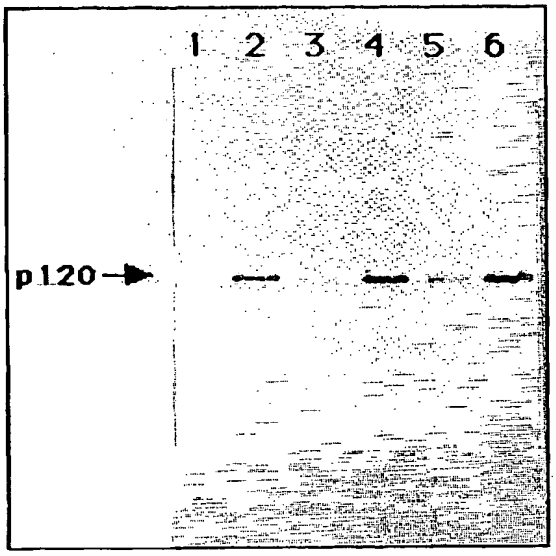

Fig. 3. The effect of p120 antisense treatment on the expression of p120 protein was determined by Western blot analysis. Lane 1, cells treated with 100 μmol/L p120 antisense oligomer; lane 2, cells treated with 100 μmol/L nonsense oligomer; lane 3, cells treated with 50 μmol/L p120 antisense; lane 4, cells treated with 50 μmol/L nonsense oligomer; lane 5, cells treated with 25 μmol/L p120 antisense oligomer; and lane 6, cells treated with 25 μmol/L nonsense oligomer.

(Fig. 4). On day 1 prior to treatment, the initial tumor volume was greater for the antisense treatment group (133 ± 101 mm^3) than the tumor volume in the nonsense treatment group (64 ± 26 mm^3). The increase in tumor growth over baseline (day 1 values; see Fig. 4, A) was calculated every 5 days. The results showed a statistical difference between the control group and the antisense-treated group. A graph for day 15 is shown in Fig. 4, *B*. Mice receiving the nonsense control oligomer grew significantly ($P = 0.0035$) larger tumors 15 days after treatment was started (average tumor volume = 425 ± 244 mm^3), as compared to p120 antisense–treated animals (average tumor volume = 166 ± 116 mm^3). In the nonsense-treated con-

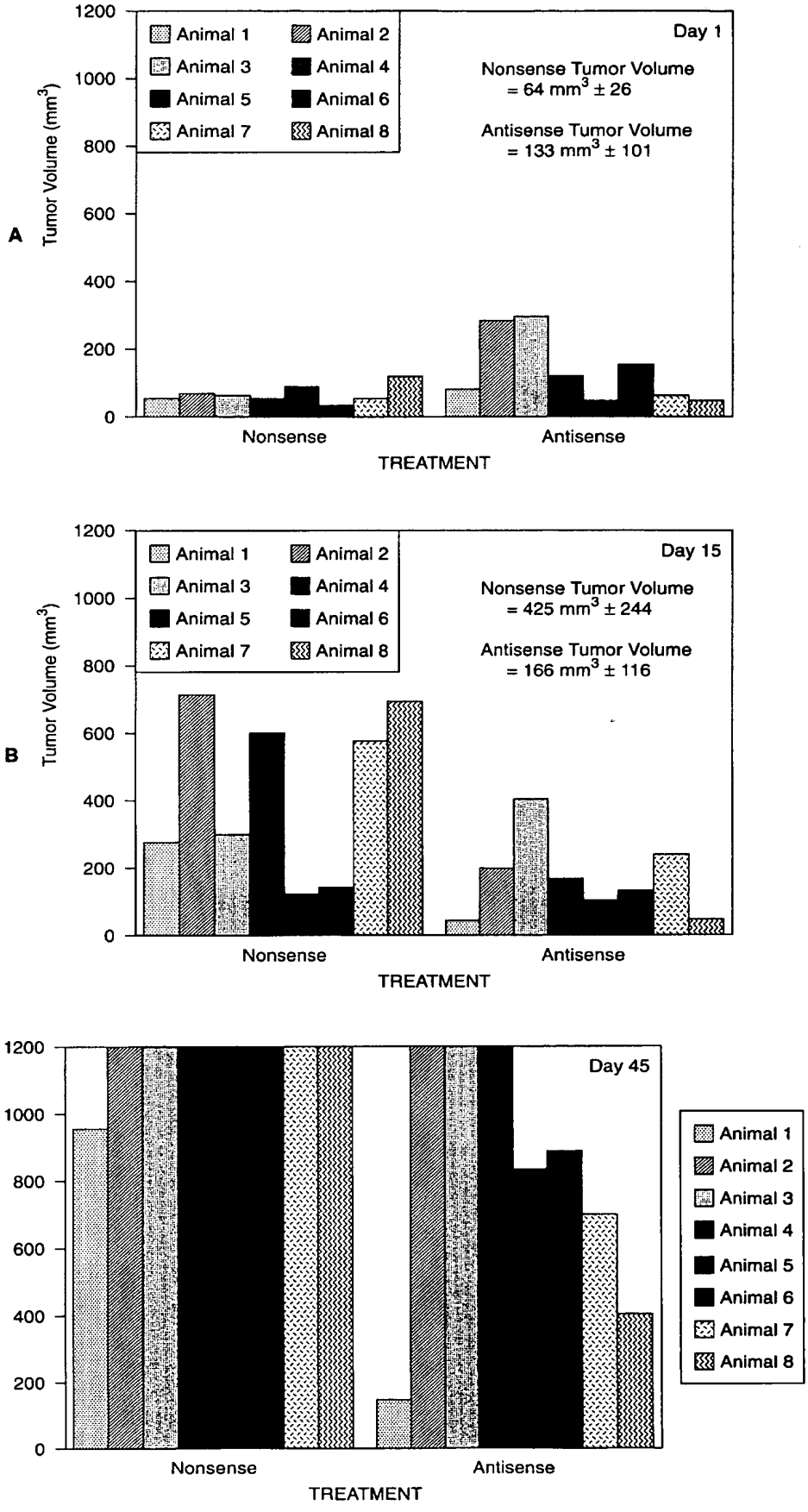

Fig. 4. For legend see opposite page.

trol group, seven of eight mice had tumors larger than 1200 mm^3 at day 45(see Fig. 4, *C*). At this same time, only three of eight mice from the p120 antisense–treated group showed tumors larger than 1200 mm^3. Interestingly, two of these three mice had rather large tumors (>150 mm^3) prior to treatment. By 60 days after treatment was started, all mice in both the control and p120 antisense–treated groups had tumors larger than 1200 mm^3.

DISCUSSION

The studies presented here evaluated the efficacy of antisense oligodeoxynucleotide therapy for inhibiting the growth of pancreatic tumor cells by blocking the specific gene expression of p120, a growth regulatory molecule. Studies using a variety of tumor types indicate the potential therapeutic efficacy of antisense oligonucleotides.[8-12]

Several potential problems exist in antisense approaches for therapy. These include the molecular target chosen and the properties of the oligonucleotide including stability, cellular uptake, and nonspecific toxicity.[7] Activated oncogenes such as *ras* are attractive targets in pancreatic cancer, but they are not expressed in all pancreatic cancers and these cancers may also have mutations of other oncogenes or tumor suppressor genes such as in p53.[19] A potentially more attractive target may be growth regulatory molecules that are downstream targets of oncogenic changes.[13,16] One such molecule having this feature is p120. p120 Plays a role in cell cycle regulation and represents a downstream target of oncogenic changes. Such oncogenic changes cause a decreased regulation in the expression of p120 at the transcriptional level.[16] Increased expression of p120 raises nucleolar function, promotes an increased proliferative potential, changes cellular morphology, and increases tumorigenic properties including a reduced dependence on normal physiologic growth regulators.[15] Conversely, a dramatic reduction in the level of p120 expression by specific antisense oligonucleotides may cause pancreatic cancer cells to undergo cell cycle arrest or cause cellular death by apoptosis or necrosis.

In this study we compared two separate p120 antisense oligodeoxynucleotides for their ability to inhibit the in vitro growth of the human pancreatic tumor cell line MIA PaCa-2. One of the antisense molecules (p120 antisense-sj) was a phosphodiester developed to overlap a splice junction of the p120 gene.[15,18] The other antisense molecule (p120 antisense-aug) was developed against the AUG transcriptional start site. The phosphodiester backbone of the latter oligomer was modified to form a phosphorothioate. These antisense molecules were first tested for their ability to inhibit DNA synthesis after a single cell culture treatment of 24 hours. Both antisense molecules inhibited DNA synthesis in a dose-dependent manner. The phosphorothioate p120 antisense aug molecule showed a greater level of inhibition at lower concentrations than the phosphodiester antisense molecule. However, the phosphodiester antisense, to the p120 splice junction, showed a somewhat greater inhibitory activity at the highest antisense concentration used (100 μmol/L). The reason for the difference in kinetics of the inhibitory activity of these two oligodeoxynucleotides is unclear from this study. These differences may reflect the difference in binding sites or the longer half-life that has been reported for phosphorothioate oligodeoxynucleotides compared to phosphodiester oligodeoxynucleotides.[7] Both antisense molecules were equally effective for inhibiting the growth of MIA PaCa-2 cells over a 7-day period. The ability of p120 antisense molecules to inhibit cell proliferation in MIA PaCa-2 cells is in agreement with our previous studies, which showed that treatment with these antisense molecules prevented cell cycle progression[13,18] and the growth of breast cancer cells in vitro.[13]

The phosphorothioate p120 antisense was chosen for in vivo studies since it showed the greatest inhibitory activity in vitro at lower concentrations and because phosphorothioates are reported to be more stable in vivo.[7,21] This antisense molecule was used in this study to determine whether it could inhibit the growth of established pancreatic tumors. The p120 antisense–treated mice showed an overall decrease in tumor size up to 45 days after treatment was begun. However, no cures were obtained and all mice developed large tumors by day 60. It is interesting to note that two of the three mice in the p120 antisense–treated group that formed large tumors at day 45 (>1200 mm^3 tumor volume) had initial tumor volumes greater than 150 mm^3. This suggests that p120 antisense may be more effective for inhibiting the growth of small tumors or micrometastasis. It remains to be determined whether an increased dose of antisense would be tolerated by the animals and whether in-

Fig. 4. Sixteen animals with palpable tumors were divided into two groups of eight animals each and treated with either nonsense or p120 antisense oligomers. Tumor volume is shown for day 1 **(A)**, day 15 **(B)**, and day 45 **(C)** after treatment was begun. The mean tumor volume is shown for days 1 and 15.

creasing the concentration of antisense would be more effective in inhibiting tumor growth. Furthermore, it is possible that multiple antisense molecules directed against other growth regulatory molecules or oncogene products would be a more effective strategy for inhibiting the growth of pancreatic tumors.

We thank Drs. Brett Monia and Frank Bennett, of Isis Pharmaceuticals, Carlsbad, Calif., for providing antisense oligomers and for helpful suggestions.

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The anticonvulsant properties of antisense c-*fos* oligodeoxynucleotides in kainic acid-induced seizures

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Abstract

Evidence has accumulated that the immediate early gene c-*fos* has important physiological and pharmacological properties in the central nervous system. The role of c-*fos* in seizures and, in particular, kainic acid-induced seizures, is unclear. It is unknown if c-*fos* stimulation after kainic acid is a consequence of neuronal activation, or an intrinsic critical component of the metabolic pathways leading to seizure. To elucidate this problem we have pretreated male Wistar rats with antisense c-*fos* and nonsense c-*fos* oligodeoxynucleotides 12 h prior to kainic acid 10 mg/kg intraperitoneal. Antisense c-*fos* inhibited the number of wet dog shakes and the appearance of limbic motor seizures, effects not seen with nonsense or vehicle. The anticonvulsant effects were associated with reduction of both Fos and NGFI-A immunoreactivity and neuroprotection in the hippocampus, thalamus and primary olfactory cortex-amygdaloid region. Four days after antisense c-*fos* limbic motor seizures were not inhibited, and there was no decrease in Fos or NGFI-A immunoreactivity and no neuroprotection, indicating that the anticonvulsant effects were not secondary to a toxic effect. Sense oligonucleotides had no anticonvulsant effects when given 12 h prior to kainic acid and did not influence immunoreactivity or neuronal survival. In conclusion, these findings suggest a role for c-*fos* in the generation of kainic acid-induced limbic seizures and neuronal death. © 1997 Elsevier Science B.V.

Keywords: Limbic seizures; Immediate early genes; Neuroprotection

1. Introduction

Manipulation of the genome offers potential therapeutic applications for human neurological diseases. In experimental animals this can be achieved in several ways: (i) by the use of transgenic animals in which a gene from another species is inserted; (ii) by knock-out animals in which a gene is disrupted or deleted; or (iii) by the use of antisense oligodeoxynucleotides (ODNs). The first two of these methods are irreversible. Antisense is reversible and permits the investigation of temporary removal of a gene, which is advantageous experimentally.

The in vitro effects of antisense ODNs were first shown to inhibit virus replication (Zamecnik and Stephenson, 1978; Zamecnik et al., 1986; Zaia et al., 1988), eukaryotic protein expression (Izant and Weintraub, 1984; Blake et al., 1985), and growth of cancer cells (Reed et al., 1990; Rosalen et al., 1990).

In 1993, two in vivo experimental animal studies revealed the ability of antisense ODNs to have effects within the CNS. Antisense of NPY1 receptors caused anxiety and selective reduction of NPY1 receptors (Wahlestedt et al., 1993b). When the NR1 subtype of glutamate receptor was antisensed, a significant decrease in

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the size of focal ischaemic infarction was found (Wahlestedt et al., 1993a). These studies demonstrated the in vivo stability and effectiveness of phosphorothioate ODNs in the brain. Phosphodiester ODNs are degraded by α-exonucleases and are stable in cerebrospinal fluid (CSF), have extensive brain penetration and are probably taken up by astrocytes (Whitesell et al., 1993). The uptake mechanism has not been defined but possibly involves a specific carrier system not involving endosomes or lysosomes (Wahlestedt, 1994).

A number of studies have reported central nervous system effects with antisense c-*fos*: inhibition of amphetamine and apomorphine-induced rotation (Chiasson et al., 1992; Dragunow et al., 1993; Hooper et al., 1994); a decrease in the motor stimulation secondary to cocaine (Heilig et al., 1993); suppression of ischaemia-induced Fos and AP-1 activity (Liu et al., 1994); disruption of circadian rhythms (Cirelli et al., 1995; Wollnik et al., 1995); and enhanced formalin-induced nociception and prodynorphin expression (Hunter et al., 1995).

It is well recognized that kainic acid (KA), given systematically to rats, induces a behavioural, electrophysiological and pathological syndrome resembling human temporal lobe epilepsy (Ben-Ari et al., 1981; Lothman and Collins, 1981; Ben-Ari, 1985). KA is also known to stimulate immediate early genes c-*fos* and c-*jun* within 30 min in limbic structures, correlating with the behavioural and electrophysiological concomitants of limbic seizures (Morgan et al., 1987; Le Galle Le Salle, 1988; Popovici et al., 1988; Sonnenberg et al., 1989a,b). KA has also been shown to elevate AP-1 DNA binding activity using gel-shift analysis (Kaminska et al., 1994; Pennypacker et al., 1994). Pretreatment with antisense c-*fos* oligodeoxynucleotides might help to elucidate the role of c-*fos* in the generation of limbic motor seizures after KA, and resolve whether c-*fos* is a consequence of neuronal activation, or a critical component of the molecular machinery leading to seizure.

Fos has a very high turnover rate in the central nervous system, is induced within minutes of stimulation, and degraded within a few hours (Sheng and Greenberg, 1990; Morgan and Curran, 1991). Therefore, substantial inhibition of Fos protein expression is predicted after a single intracerebroventricular injection of antisense c-*fos*, and this is the experimental approach which has been used.

2. Experimental procedures

2.1. Animals

Male Wistar rats weighing 200–300 g were obtained from B&K Universal Ltd. They were housed with 5–6 animals per cage. Food and water were freely available. The animal rooms were maintained on a 12-h light/dark cycle. All efforts were made to minimize animal suffering and to reduce the numbers of animals used.

2.2. Stereotaxic cannulation of the cerebral ventricles

Male Wistar rats (200 g) were anaesthetized with HYPNORM (Janssen, UK) 0.2 ml/kg i.m. (fentanyl 0.315 mg+fluorisone 10 mg/ml) and diazepam 2.5 mg/kg i.p. The top of the head was shaved and the animal placed in a Kopf stereotaxic frame. The following coordinates were used relative to the midpoint of the interaural line:

anterior − posterior = + 7.7 *mm*

lateral = + 0.6 *mm*

dorsoventral = + 6.4 *mm*

The coordinates were developed for 200-g animals and based on those of Paxinos and Watson (1982). Initial experiments showed accurate placement of the guide cannula in 90% of animals using Evan's blue. At the conclusion of each experiment, the guide cannula tip was checked visually; if the position was incorrect, the animals were excluded.

Skull holes were made with a dental drill. The guide cannula and support screws were held in place using dental cement. At the conclusion of the procedure each animal was given: 2 ml of glucose/saline i.p.; naloxone 1 mg/kg i.p.; and penicillin 50 μg i.m. The animals were placed on a homeothemic blanket at 37°C until recovery, and water was freely available. After 7 days the antisense experiments were performed.

2.3. Antisense and other oligodeoxynucleotide probes

Phosphorothioate ODN analogues of antisense c-*fos*, nonsense c-*fos*, and sense c-*fos* were prepared using an Applied Biosystems Synthesizer (Model 392) — Table 1.

Table 1
Phosphorothioate analogues used in the antisense experiments

Gene	Sequence 5′-3′	No. of nucleotides	Base composition				Nucleotide position in gene/homology	References
			A	C	G	T		
Antisense c-*fos*	GAA CAT CAT GGT CGT	15	4	3	4	4	129-143 rat c-*fos* cDNA	Chiasson et al., 1992
Sense c-*fos*	ACG ACC ATG ATG CTT	15	4	4	3	4		Curran et al., 1987
Nonsense c-*fos*	ATA GAA GCT CCT CTG	15	4	4	3	4	No homology to any rat gene	GenBank

Probe concentration was adjusted to 50 μg/5 μl in physiological saline.

The ODNs were given i.c.v. using an injection cannula which extended 1 mm ventral to the guide tip. Probe solutions were injected with a microlitre syringe (Hamilton, USA) at a rate of 1 μl/min.

2.4. Drugs

Kainic acid (KA, 2-carboxy-4-(1-methylethanyl)-3-pyrrolidinacetic acid) was obtained from Research Biochemicals International, USA and dissolved in physiological saline and adjusted to pH 7.4.

2.5. Experimental design

Antisense and other ODNs were given 10 h prior to KA 10 mg/kg i.p. unless otherwise stated. This time point was chosen because in other systems behavioural effects were observed at this time and using the same sequence of antisense c-*fos* (Chiasson et al., 1992; Hooper et al., 1994). KA was always given in the morning to avoid possible diurnal effects on AP-1 stimulation (Unlap and Jope, 1995).

After KA, animals were individually placed in clear perspex boxes (30×30×30 cm), the back of the box had a mirror as an aid in measuring behaviour. The number of wet dog shakes (WDS) in the first hour and the time to onset of limbic motor seizures were measured. Limbic motor seizures (LMS) were defined by the simultaneous onset of a striking syndrome characterized by salivation, forelimb myoclonus, and rearing on the hindlimbs. At the conclusion of the behavioural observations, rats were placed in separate holding cages until perfusion fixation.

Analysis of variance (ANOVA) followed by the Tukey-Kramer post-test were used to examine differences between multiple treatment groups with respect to numbers of animals, and time to onset of WDS and LMS.

2.5.1. Perfusion fixation

Animals were given pentobarbitone 30 mg i.p. and heparin 50 IU in 0.1 ml normal saline i.p. When anaesthetized, a thoracotomy and laparotomy were performed. The descending aorta was clamped before inserting a 21G needle into the left ventricle. This needle was connected to two reservoirs 1 m above the operating table. The first infusion of 0.9% saline (Galen) lasted 5 min and was followed by 5 min of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS) pH 7.4.

2.6. Immunocytochemistry

Perfusion fixation was performed 6 h after KA for all antisense experiments. The brains were placed in 4% PFA at 4°C for 24 h, and then in 20% sucrose in PBS for another 48 h.

A freezing microtome was used to cut 40 μm sections which were incubated overnight with primary antibody (Fos at 1:4000 or NGFI-A at 1:1000, both provided by Drs G. Evan and D. Hancock, ICRF, London). Biotinylated anti-rabbit antibody (1:200) was then applied for 1 h. After a wash in PBS, avidin-biotin-horseradish peroxidase complex was used at 1:200. The reaction product was developed using diaminobenzidine.

Fos and NGFI-A positive nuclei were counted manually in 10 high-power fields in CA1. The results were expressed as positive nuclei per 10^4 μm^2. Data were analyzed using a *t*-test, or ANOVA followed by the Tukey-Kramer post-test if there were more than two experimental groups.

2.6.1. Staining

Blocks were mounted in cassettes and paraffin embedded 10 days after perfusion fixation, the brains having been in 4% PFA until mounting. Sections 8 μm thick were cut on a microtome and floated onto slides. They were stained with H&E, Luxol fast blue/cresyl fast violet and a silver stain. Histological damage was assessed semiquantitatively using a 0–3 point scale without knowledge of the experimental treatment: 0=no damage, cell loss or infiltration; 1=mild damage, cell loss, infiltration; 2=moderate damage, cell loss, infiltration; 3=severe damage, cell loss, infiltration (as used by Friedman et al. (1994) and Kesslak et al. (1995), for KA-induced damage).

3. Results

3.1. The effects of antisense and nonsense

Antisense c-*fos* reduced the number of WDS in comparison with nonsense and vehicle (Table 2). Only two out of six antisense-treated animals developed LMS with a latency to onset of 96.5±5.5 min, compared to 70.5±2.9 min for nonsense and 67.8±2.0 min for vehicle. (ANOVA $F=73.51$, $P=0.005$, extremely significant; post-test comparisons between antisense and other groups $P<0.001$). All animals that received nonsense and vehicle developed LMS (Fig. 1). Antisense c-*fos* significantly reduced the number of Fos positive nuclei in the CA1 region: antisense 2.4±0.7 vs. 33.0±3.1 for nonsense ($\bar{x}$±ISE, $n=3$, *t*-test $t=22.47$, $P=0.0007$). Similarly the NGFI-A positive nuclei were also reduced: antisense 6.9±1.0 vs. 36.2±6.4 (*t*-test $t=10.35$, $P=0.011$) — Fig. 2.

3.2. The effects of 'washout'

When antisense and nonsense were given 4 days prior to KA, the numbers of WDS were less in the antisense-treated group (Table 2). All treatment groups developed LMS, and there was no delay in their onset (Fig. 3). After 'washout' there was no difference in the number of Fos positive nuclei in CA1: antisense 39.9±1.1, nonsense

Table 2
The effects of antisense and other oligodeoxynucleotides on wet dog shakes after kainic acid

	Experimental treatment		Wet dog shakes in first hour after kainic acid 10 mg/kg ($\bar{x}\pm$ISE, $n=6$)
1.	The effects of antisense and nonsense		
	Antisense		42.5±20
	Nonsense		150±30
	Vehicle		115±10
2.	The effects of 'washout'		
	Antisense		72±10
	Nonsense		150±25
	Vehicle		146±25
3.	The effects of sense and antisense		
	Antisense	50 mg	17±5
	Antisense	100 mg	15±6
	Sense	50 mg	92±25
	Nonsense	50 mg	125±10
	Vehicle		90±20

38.8±2.3, vehicle control 42.4±1.4 (ANOVA $F=5.84$, $P=0.363$). The number of NGFI-A positive cells was also not affected: antisense 38.3±1.5, nonsense 40.5±1.8, vehicle 43.3±2.5 (ANOVA $F=9.25$, $P=0.274$).

3.3. The effects of sense

Sense c-*fos* given 12 h before KA had no effect on the number of WDS in comparison to antisense at doses of 50 and 100 μg (Table 2). All animals that received sense developed LMS without delay in their onset. LMS were observed in two out of six animals given 50 μg of antisense c-*fos* and in one out of six animals given 100 μg. LMS were delayed after both treatments (Fig. 4). Sense pretreatment did not affect the number of Fos positive nuclei: sense 41.9±2.0, antisense 0.70±0.2, nonsense 38.5±2.4 (ANOVA $F=426.33$, $P<0.0001$). Similarly, sense pretreatment did not influence the number of NGFI-A positive cells: sense 43.8±2.0, antisense 3.60±0.4, nonsense 35.7±2.8 (ANOVA $F=498.54$, $P<0.0001$).

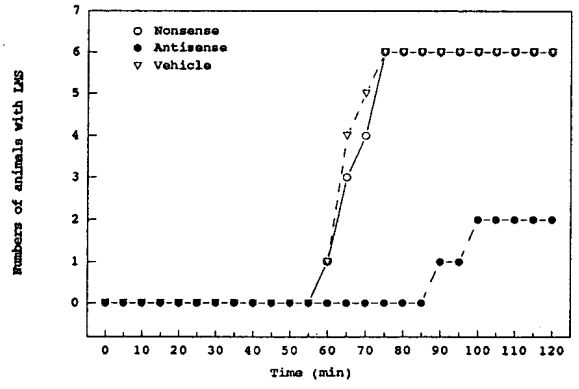

Fig. 1. The effects of antisense and nonsense oligodeoxynucleotides on kainic acid-induced limbic seizures. Animals given kainic acid 10 mg/kg i.p. preceded 12 h before by 50 μg i.c.v. of antisense or nonsense oligodeoxynucleotides. Vehicle-tested animals given 5 μl of physiological saline. LMS, limbic motor seizures.

3.4. Neuronal injury

Antisense c-*fos* at 50 and 100 μg protected neurons in the CA1 region of the hippocampus, dorsal thalamus (DThN), and in the primary olfactory cortex-amygdaloid region (POCx-AMYG). Pretreatment with nonsense, sense and vehicle did not prevent neuronal death after KA (Table 3).

4. Discussion

The major finding from these series of experiments is that antisense c-*fos* had anticonvulsant properties, and inhibited limbic seizures. KA-induced seizures are associated with electrical discharges arising from the hippocampus and other structures within limbic circuitry (Ben-Ari et al., 1981; Ben-Ari, 1985) and c-*fos* stimulation in these same structures (Morgan et al., 1987; Le Galle Le Salle, 1988; Popovici et al., 1988; Sonnenberg et al., 1989a,b). The results presented in this study suggest that Fos protein is of importance in the generation of limbic seizures. That is, Fos is not a consequence of seizures; and the inhibition of both Fos and NGFI-A immunoreactivity after antisense treatment strongly supports this possibility.

The 'washout' experiment provides evidence against any toxic effects of antisense c-*fos*, as anticonvulsant effects had dissipated within 4 days after injection. At this time there was no difference between antisense-, nonsense- and vehicle-treated animals. The 'washout' experiment also suggests that by metabolic clearance of antisense c-*fos* from the brain, its pharmacological and physiological effects had also worn off. This is compatible with published studies showing clearance of phosphorothioate ODNs within 48 h (Whitesell et al., 1993).

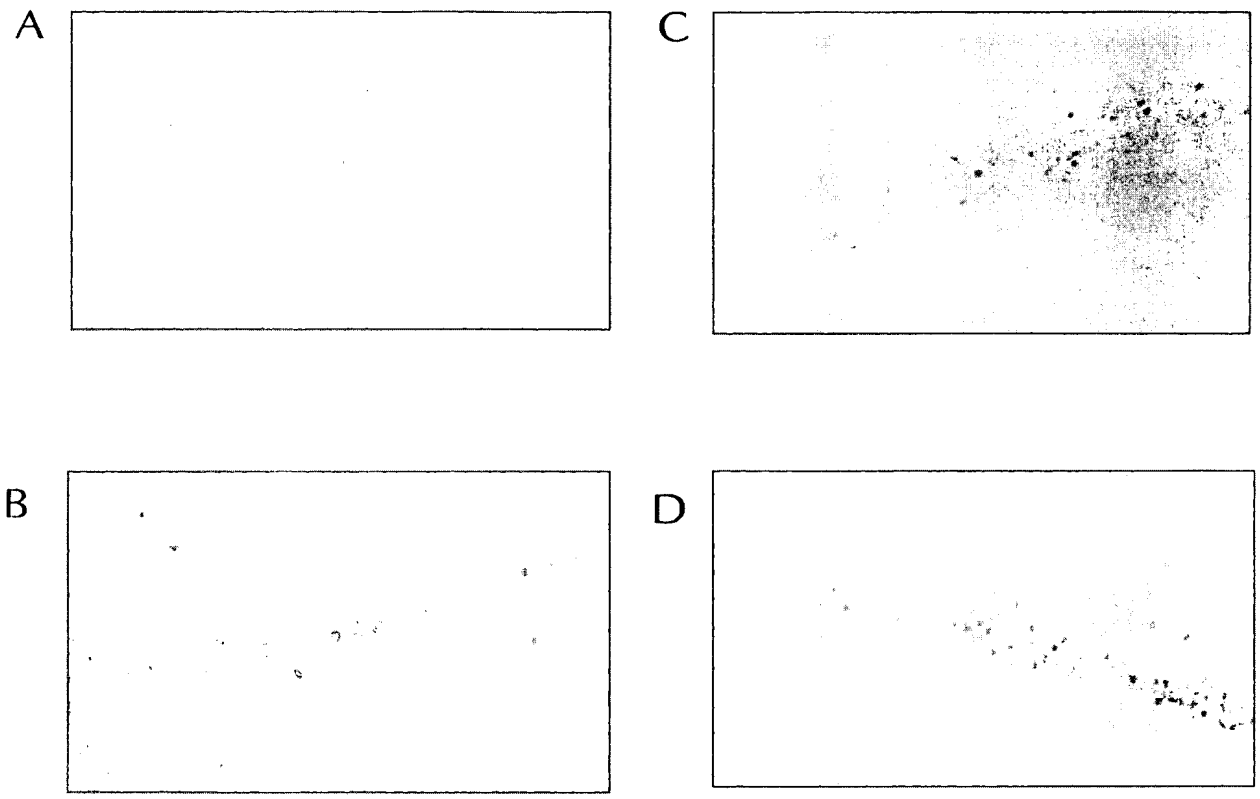

Fig. 2. The effects of antisense and nonsense oligodeoxynucleotides on Fos and NGFI-A immunoreactivity after kainic acid in the CA1 field of the hippocampus. Antisense and nonsense given 12 h prior to kainic acid (10 mg/kg i.p.). Perfusion fixation done 6 h after kainic acid. Fos IR after antisense and sense (A and B, respectively). NGFI-A IR after antisense and nonsense (C and D, respectively) (×320).

ODNs may have non-antisense effects because: (i) they are charged polyanions, and (ii) they might have sequence-selective non-antisense effects. These two possibilities seem unlikely in the context of the present series of

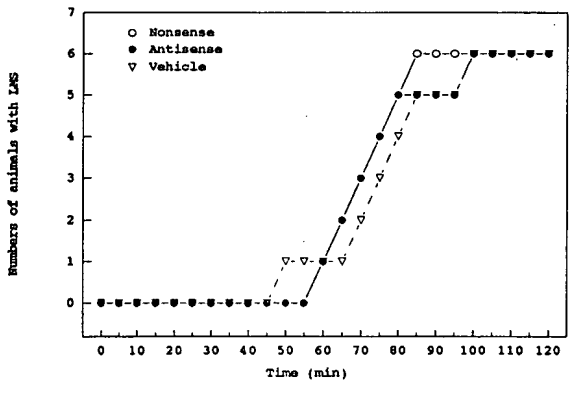

Fig. 3. The effects of 'washout' of antisense and nonsense oligodeoxynucleotides on kainic acid-induced limbic seizures. Animals given antisense, nonsense or vehicle 4 days prior to kainic acid 10 mg/kg i.p. LMS, limbic motor seizures.

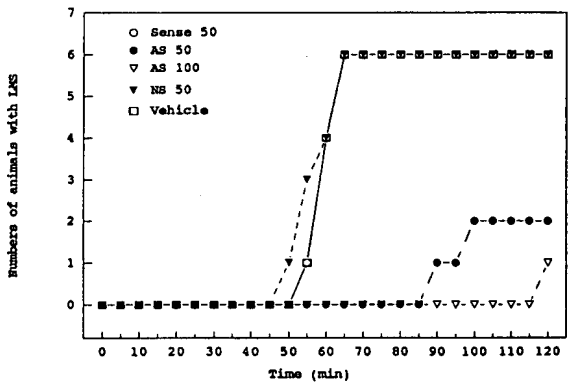

Fig. 4. The effects of sense and oligodeoxynucleotides antisense dose on kainic acid-induced limbic seizures. Oligodeoxynucleotides (ODN) and vehicle given 12 h before kainic acid 10 mg/kg i.p. Sense 50, 50 μg of sense ODN; AS 50, 50 μg of antisense ODN; AS 100, 100 μg of antisense ODN; NS 50, 50 μg of nonsense ODN; vehicle, 5 μl of physiological saline. LMS, limbic motor seizures.

Table 3
The histological effects of pretreatment with c-*fos* antisense and other oligodeoxynucleotides on kainic acid-induced neuronal death[a]

Animal treatment	No.	CA1	DThN	POCx-AMYG
c-*fos* anti-sense 50 μg i.c.v.	1	0	0	0
	2	2	2	2
	3	0	0	0
	4	1	1	1
	5	0	0	0
	6	0	0	0
c-*fos* antisense 100 μg i.c.v.	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0
	6	0	0	0
c-*fos* nonsense 50 μg i.c.v.	1	3	3	3
	2	3	3	3
	3	3	3	3
	4	2	2	2
	5	3	3	3
	6	1	2	1
c-*fos* sense 50 μg i.c.v.	1	3	3	3
	2	3	3	3
	3	2	2	2
	4	2	2	2
	5	3	3	3
	6	3	3	3
Vehicle 5 μl i.c.v.	1	3	3	3
	2	3	3	3
	3	2	2	3
	4	3	3	3
	5	3	3	2
	6	2	2	3

Antisense oligodeoxynucleotides given 10 h prior to kainic acid.
[a]Dose of kainic acid = 10 mg/kg i.p. Animals killed 10 days after KA for histology.
Histological scale of death from 0 = no neuronal death/cell infiltration/reaction to 3 = maximal neuronal death and cell infiltration/reaction.

experiments since sense and nonsense sequences had no anticonvulsant effects. Also, the nonsense sequence was made of the same base composition as the antisense sequence, but in random order, and it too had no effects. Recently, some of the previously reported antisense effects of phosphorothioate analogues in other experimental systems have been attributed to four tandem guanosine residues — the G-quartet (Burgess et al., 1995). The antisense c-*fos* sequence used in this study does not contain the guanosine set.

Despite the molecular target being c-*fos* mRNA, binding of antisense c-*fos* to other genes, proteins, or receptors cannot be excluded, and might contribute to some of the effects — no matter how specific the sequence is. It is also possible that cells in the mammalian CNS respond to DNA through hybridization-independent mechanisms, as has recently been shown for mammalian lymphocytes (Krieg, 1995).

The immunocytochemical results reveal a reduction in both Fos and NGFI-A in antisense-treated animals. This correlated with the inhibition of LMS. The inhibition of both of these genes is not surprising given the anticonvulsant properties of antisense c-*fos*. If antisense c-*fos* was not anticonvulsant, both genes would be elevated. Given the experimental system used here to explore the relationship between c-*fos* and seizure, a differential expression would not be expected because of the important role of Fos in the generation of limbic seizures. It is also possible that Fos influences the expression of NGFI-A, such that downregulation of one gene might affect the other. Other studies using the same sequence of antisense c-*fos* have also found reductions in other immediate early genes. A study of amphetamine-induced rotation showed the inhibition of Fos after antisense c-*fos* and the NGFI-A results were not presented (Chiasson et al., 1992). A time-dependent decrease in both Fos and NGFI-A was found after antisense c-*fos* in another study of amphetamine-induced rotation (Hooper et al., 1994). Reductions in both Fos and JunB were found when both apomorphine and amphetamine were studied (Dragunow et al., 1993). The immunocytochemical effects of antisense c-*fos* in cocaine-induced locomotor stimulation were not described (Heilig et al., 1993). Therefore, the relationship between antisense c-*fos* and immunoreactive changes in immediate early genes remains unclear from the published literature, but suggests that antisense c-*fos* might also influence other immediate early genes. Possibly the more fundamental the role of Fos in a particular experimental situation involving neuronal activation, like seizures and drug-induced rotation, the more likely other immediate early genes will be affected.

The mechanism of antisense effect possibly relates to translational arrest or substrate provision for RNase H, which breaks down mRNA. Both mechanisms might be operative, and further studies may elucidate which of these mechanisms is more likely. Detection of ^{32}P-labelled antisense c-*fos* in the nucleic acid fraction, and treatment of the recovered nucleic acids with RNase H would indicate an association of the antisense ODN with RNA and cellular uptake (Liu et al., 1994). RNase protection assays followed by Northern analysis might also assay the incorporation of antisense c-*fos* into RNA.

Fos, in addition to its other putative functions, is considered to regulate the expression of late onset genes through interactions at the AP-1 site. The time course of anticonvulsant effects secondary to antisense c-*fos* suggests that this mechanism is unlikely to be operative through changes in late onset genes, and further supports the possibility of a fundamental role for Fos in limbic seizure generation, possibly through membrane, receptor, or neurotransmitter-like effects. Such effects are probably also important in the basal ganglia.

In addition to producing limbic seizures, KA induces neuronal death in limbic structures (Ben-Ari et al., 1981; Lothman and Collins, 1981; Ben-Ari, 1985). Anticonvulsants which prevent the development of limbic seizures also protect neurons (Ault et al., 1986; Lees, 1992). The mechanism of neuronal death probably relates to continu-

ing seizures activity and consequent elevations in intracellular Ca^{2+}. Elevated intracellular Ca^{2+} levels are also thought to contribute to neuronal death in other pathological situations like ischaemia (Choi, 1988; Choi and Rothman, 1990). KA releases presynaptic glutamate and the subsequent elevation in intracellular Ca^{2+} is another mechanism by which neurons might be injured (Coyle, 1983; Ferkany and Coyle, 1983). Antisense c-*fos* inhibited LMS and this probably accounts for the neuroprotective effects. Studies using a *fos-lac* Z transgenic mouse suggested that c-*fos* might be involved in programmed cell death in development and after KA (Smeyne et al., 1993). An additional effect of antisense c-*fos* through suppression of programmed cell death is a possibility, but thought unlikely — even though apoptosis and delayed neuronal death probably contribute to neuronal death in hippocampal neurons after KA (Pollard et al., 1994).

5. Conclusions

Antisense c-*fos* inhibited limbic motor seizures after KA, decreased Fos and NGFI-A immunoreactivity, and protected neurons in limbic structures. These effects were not seen with sense, nonsense, or vehicle pretreatments. These findings suggest a role for c-*fos* in the generation of limbic seizures.

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ELSEVIER

ANTIPROLIFERATIVE EFFECTS OF c-myc ANTISENSE OLIGONUCLEOTIDE IN PROSTATE CANCER CELLS: A NOVEL THERAPY IN PROSTATE CANCER

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ABSTRACT

Objectives. To explore the possibility of using antisense oligonucleotide therapy for prostate cancer, we investigated the effect of *c-myc-antisense-oligonucleotide (c-myc-As-ODN)* in human prostate cancer cell lines such as LNCaP, PC3, and DU145.

Methods. LNCaP, PC3, and DU145 cells were incubated in the presence of *c-myc-As-ODN*. Dose (0 to 10 μM) and time dependent (1 to 6 days) effects on proliferation and viability were examined by [^{3}H]thymidine incorporation and MTT assay, respectively. Flow cytometry analysis was carried out to analyze cell cycle status by determining the DNA content in LNCaP cells. Control cultures received either *c-myc-sense-ODN* or scrambled (nonsense) nucleotides.

Results. Time- and dose-dependent decreases in DNA synthesis and cell viability were noted for all three prostate cancer cell lines after *c-myc-As-ODN* treatment. Further studies using LNCaP cells indicated that these changes were accompanied by an increase in the percentage of cells with less than 2N DNA content after *c-myc-As-ODN* treatment. The results suggest that *c-myc-As-ODN* induces cell death. Comparison of a *c-myc-As-ODN*-treated group with a group subjected to isoleucine deprivation revealed that thymidine incorporation was almost the same in *c-myc-As-ODN*-treated LNCaP cells and in LNCaP cells at early S phase.

Conclusions. These results suggest that *c-myc-As-ODN* inhibits prostate cancer cell growth and proliferation mainly by decreasing cell viability.

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The *c-myc* protooncogene encodes for a *c-myc* nucleoprotein, which plays a central role in cell proliferation and differentiation.[1] *C-myc* is an early gene, the expression of which is induced by various mitogens.[2] That *c-myc* is involved in the signal transduction pathways leading to cell division is suggested by several lines of evidence derived from studies of either transformed cell lines, blood cell lines, or transgenic mice.[3] Such studies have demonstrated that proliferating cells have higher levels of *c-myc* mRNA and *c-myc* encoded protein than do quiescent cells, which remain constant throughout the cell cycle.[4] *C-myc* mRNA is markedly induced upon stimulation of resting cells by mitogens to pass from G0 to G1.[5] In complementary experiments, *c-myc* RNA levels fall dramatically when cells withdraw from the cell cycle into G0 or undergo terminal differentiation.[6] Antibodies directed against the human *c-myc* protein inhibit DNA synthesis of nuclei isolated from human cells; conversely, constitutive expression of *c-myc* produced by gene transfer inhibits induced differentiation of several cell lines and predisposes to the development of tumors in transgenic mice.

Overexpression of *c-myc* has been demonstrated in a number of benign and malignant cell lines, including prostate cancer.[7,8] Prostate cancer is the most commonly diagnosed cancer in men in the United States, and it was estimated that 317,100 new cases would be diagnosed and 41,400 men would die of the disease in 1996.[9] Patients with metastatic prostate cancer initially respond to hormonal therapy, but a significant proportion of

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TABLE I. *Sequence of 15-mer oligonucleotides used in this study

c-myc antisense oligonucleotide	5'-AAC GTT GAG GGG CAT-3'
c-myc sense oligonucleotide	5'-ATG CCC CTC AAC GTT-3'
c-myc nonsense oligonucleotide	5'-GAA CGG AGA CGG TTT-3'

* *Data obtained from the Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts.*

these patients become resistant to hormonal therapy and die of the disease. At the current time, there is no effective treatment for these patients. Gene therapy, targeting specific genetic deregulation, may prove to be an effective therapeutic strategy in prostrate cancer. Antisense oligonucleotides, targeted against several oncogene mRNAs, have been shown to inhibit cell proliferation in many benign and malignant cells.[10] We investigated the effect of *c-myc-As-ODN* on LNCaP cells, an androgen-dependent, human prostate cancer cell line, and PC3 and DU145, androgen-independent human prostate cancer cell lines. To the best of our knowledge, this is the first in vitro study to demonstrate that *c-myc-As-ODN* therapy may be useful in the control of prostate cancer growth.

MATERIAL AND METHODS

Cell Culture

Androgen-dependent human metastatic prostrate cancer cell line from lymph nodes (LNCaP)[11] and androgen-independent metastatic prostate cancer cell lines from bone (PC3)[12] and brain (DU145)[13] were obtained from the American Type Culture Collection (Bethesda, Md). These cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air and serially passaged in RPMI medium, supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). In addition, LNCaP cells received testosterone (10^{-7} M).

Phosphorothioate Oligonucleotides

C-myc oligonucleotides were synthesized using a DNA synthesizer model 396 (Applied Biosystems, Foster City, Calif), purified through CEPACK columns (Millipore, Milford, Mass), lyophilized overnight, and suspended in DEPC-treated distilled water.[14] The sequences of antisense, sense, and nonsense oligonucleotides used in our experiments are shown in Table 1.[15]

MTT Assay for Cell Growth and Viability

Cell growth and viability were tested by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrozolium bromide) (Sigma Chemical Co.) assay.[16] LNCaP, PC3, and DU145 cells were plated (5000 cells/well) in 96 well plates. After 48-hour incubation in RPMI medium containing 10% serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and/or testosterone (10^{-7} M) at 37°C, the cells were serum starved overnight. To minimize antisense oligonucleotide turnover due to serum components, cells were first deprived of serum and then allowed to grow in medium containing 2.5% serum. For the time-course experiments, cells were exposed to 5 μM antisense oligonucleotide or sense oligonucleotide for 6 hours, followed by addition of 2.5% serum, and the incubation was continued for 1, 4, and 6 days. We used 2.5% serum in our experiments instead of 10% serum because in our preliminary experiments both groups produced identical results. MTT (5 mg/mL) was prepared in phosphate-buffered saline (PBS) and 20 μL was added to each well. Each concentration or time point was run in replicates of 6 or 8 wells. The cells were incubated for 4 hours at 37°C. The medium was removed at the end of incubation, dimethyl sulfoxide (200 μL) was added to each well, and optical density (OD) was read at 515 nm. Experiments were carried out in a similar fashion for dose-response studies after exposing the cells to *c-myc-As-ODN* (0.25 to 5 μM) in RPMI medium containing 2.5% serum. Control cultures received 2.5% serum only. The cells were incubated for 3 days.

[^{3}H]Thymidine Incorporation. LNCaP, PC3, and DU145 cells were plated (2×10^3 cells/well in 12 well plates) as described above. After a 48-hour incubation at 37°C, the cells were serum starved for 16 hours and exposed to *c-myc-As-ODN* (0.25 and 10 μM) or sense oligonucleotide (10 μM) for 6 hours before the addition of 2.5% serum. Control cell cultures contained 2.5% serum. The incubation was continued for 3 days and during the last 6 hours of incubation, 1 or 2 μCi/mL of [^{3}H]thymidine (Dupont NEN Products, Boston, Mass) mixed with 0.1 mM cold thymidine was added to each well to be incorporated into DNA.[17] At the end of incubation, the medium was removed, the wells were rinsed twice with PBS, pH 7.4, and trypsinized with 500 μL of trypsin ethylenediaminetetraacetic acid for 30 to 45 minutes at 37°C. The cell suspension was combined with equal amounts of 10% trichloroacetic acid (TCA) and the acid-insoluble material was collected on Whatman glass fiber filters. The filters were dried and counted in 10 mL of OFTI-FLOUR scintillation fluid (Packard Instrument Company, Meriden, Conn).

Autoradiographic Detection of Labeled Nuclei

LNCaP cells were plated (10^4/3.5 cm culture dishes) and incubated for 48 hours in RPMI medium containing 10% serum prior to overnight serum starvation. The cells were then exposed to antisense (0.25 to 5 μM), sense (5 μM), or nonsense oligonucleotides (5 μM) followed by the addition of 2.5% serum. The medium was removed at the end of 3 days of incubation, and the cells were pulsed with [^{3}H]thymidine (3 μCi/mL) for 60 minutes. The cells were washed twice with ice-cold PBS, pH 7.4, before fixation in methanol/acetic acid (2:1) for 30 minutes. They were then washed twice with methanol and air dried. A thin film of Kodak nuclear track emulsion (NTB 3) was added to each plate, and the plates were developed following 4 to 5 days of exposure in the dark. Cells were counter-stained with Geimsa and the labeled nuclei were further classified into densely labeled (representing a rapidly proliferating nature of the cells), stippled (indicating relatively slower incorporation of thymidine into DNA), and unlabeled nuclei (representing nondividing or dead cells). The number of densely labeled, stippled, and unlabeled nuclei were counted under the microscope.

Flow Cytometry

LNCaP cells were plated (3×10^4 cells/well in 6 well plates) in 10% serum containing RPMI medium. After a 48-hour incubation at 37°C, 5% CO_2/95% air, the cells were serum starved for 16 hours. The cells were exposed to antisense oligonucleotide (5 μM), or sense oligonucleotide (5 μM), or nonsense oligonucleotide (5 μM) for 6 hours followed by addition of 2.5% serum. The cells were

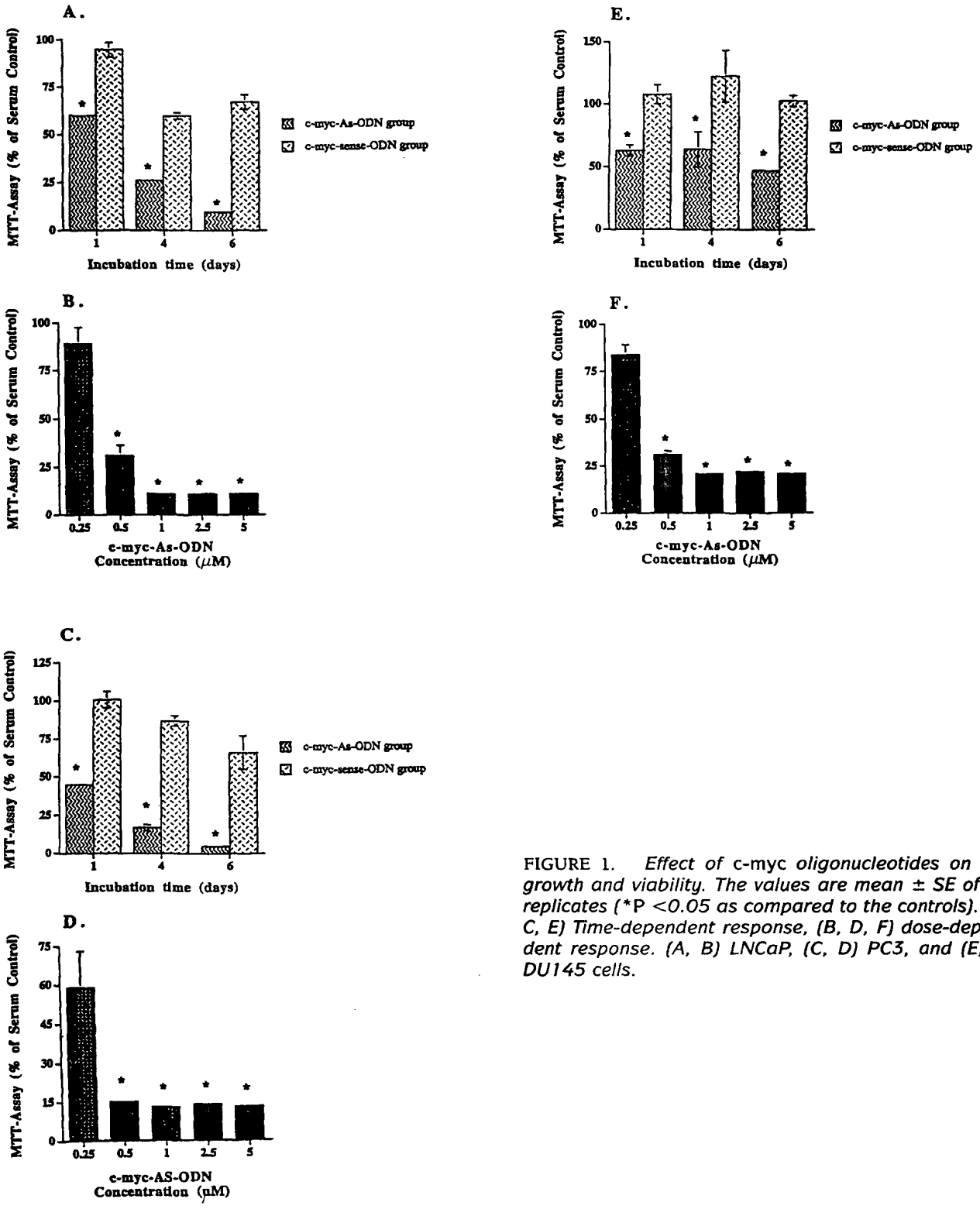


FIGURE 1. *Effect of* c-myc *oligonucleotides on cell growth and viability. The values are mean ± SE of six replicates (*P <0.05 as compared to the controls). (A, C, E) Time-dependent response, (B, D, F) dose-dependent response. (A, B) LNCaP, (C, D) PC3, and (E, F) DU145 cells.*

trypsinized at 0, 24, 48, and 96 hours, washed with PBS, fixed in 70% ethanol, treated with extraction buffer (0.05 M Na_2HPO_4 and 25 mM citric acid at a 9:1 ratio, 0.1% Triton X-100 (Sigma) for 10 to 20 minutes at room temperature, resuspended in 10 mM piperazine-*N*,*N*′bis-(2-ethanesulfonic acid) (PIPES) buffer containing 0.1 N NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, pH 6.8, 20 μg/mL of propidium iodide, and 50 μg/mL of RNase for 30 minutes at room temperature,[18] and run on FACS scan (Becton Dickinson Immunocytometry Systems, San Jose, Calif). The data were

analyzed using Winlist and Modfit computer software programs (Verity Software House, Topsham, Me).

Synchronization of LNCaP Cells

LNCaP cells were plated in 35-mm dishes (5×10^4 to 6×10^4) and allowed to grow in RPMI-1640 medium containing 10% fetal bovine serum (FBS), antibiotics, and 10^{-7} M testosterone. Once the cells were attached, they were allowed to grow in isoleucine-free medium containing 6% dialyzed FBS for 48 hours.[19-21] At the end of the isoleucine deprivation period, they were changed to complete medium containing 10% serum. Thymidine (4 μCi/plate) incorporation was carried out at time intervals of 3 hours up to 18 hours.

Statistical Analysis

The data were analyzed using one-way analysis of variance with a significance level of 0.05 (Mystat statistical software program, SPSS Inc.).

RESULTS

In our studies, we used antisense oligonucleotide targeted against the translation start site of *c-myc* protooncogene. Sense and scrambled (*nonsense*) oligonucleotides were used as controls (Table I).

Initially, to evaluate the effect of *c-myc* oligonucleotides on the cell growth and viability, MTT assay was done. It showed a time-dependent, statistically significant decrease in cell viability in the *c-myc-As-ODN* treated groups (Fig. 1A,C,E). A slight decrease in cell viability was noted in sense oligonucleotide treated PC3 and LNCaP groups, and it was not statistically significant as compared to that in the respective control groups ($P > 0.05$). There was no change in viability in the sense oligonucleotide treated DU145 cells. The effect of antisense oligonucleotide treatment on cell viability was also dose-dependent (Fig. 1B,D,F). A decrease in cell viability was noted at doses as low as 0.5 μM *c-myc-As-ODN*.

The inhibitory effect of the antisense oligonucleotide on MTT assay may be due to decreased cell viability and/or decreased cell proliferation. To evaluate the specific effect on cell proliferation, we determined [^{3}H]thymidine incorporation into DNA. Log phase LNCaP, PC3, and DU145 cell cultures were used for these studies. Treatment with *c-myc-As-ODN* decreased [^{3}H]thymidine incorporation in a concentration-dependent manner (Fig. 2), with a reduction of 50% (LNCaP), 72% (PC3), and 30% (DU145) at 10 μM ($P < 0.05$) and no reduction at 0.25 μM concentration. *C-myc-sense-oligonucleotide,* at 10 μM concentration, caused 40% and 30% reduction in thymidine incorporation by LNCaP and PC3 cells and it was statistically significant ($P < 0.05$). There was no effect of *c-myc-sense-oligonucleotide* on thymidine incorporation by DU145 cells.

Autoradiographic detection of nuclear labeling with [^{3}H]thymidine was carried out using 5 μM

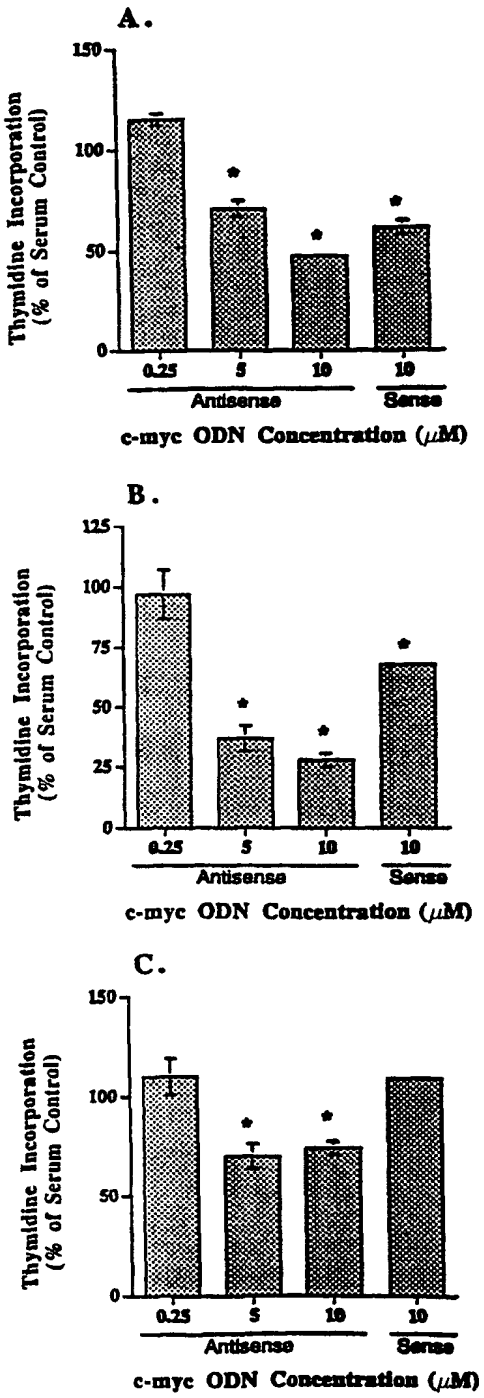
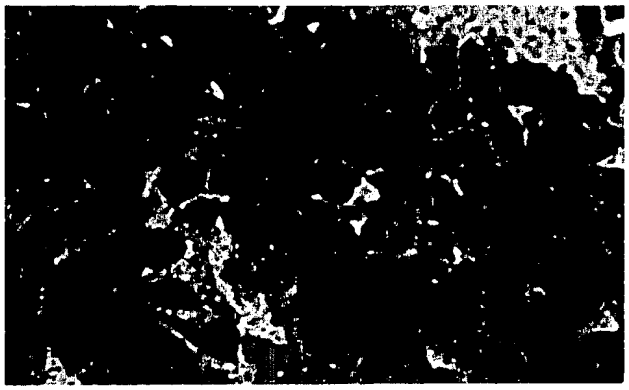


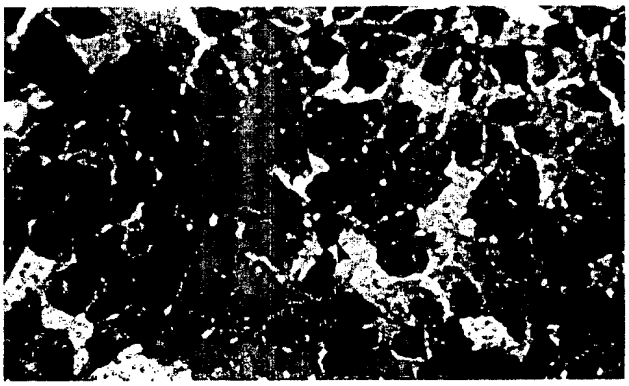
FIGURE 2. *Effect of* c-myc-*oligonucleotides on [^{3}H]thymidine incorporation into DNA. The values are mean ± SE of six replicates (*P <0.05). (A) LNCaP, (B) PC3, (C) Du145 cells.*

c-myc-antisense or *sense* or *nonsense* oligonucleotides and LNCaP cells.

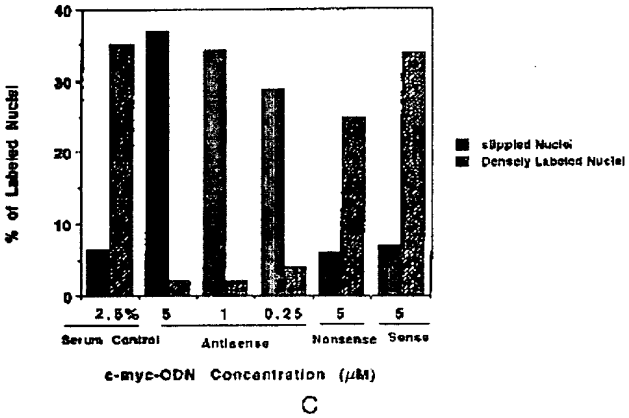
Representative pictures of LNCaP cells showing the dense and stippled nuclear labeling in serum control and *c-myc-As-ODN* treated groups are given (Fig. 3A,B). The numerical analysis of the



A



B



C

FIGURE 3. *(A, B) Representative pictures showing stippled (single arrow) and dense nuclear labeling (double arrow) of [^{3}H]thymidine into DNA: (A) serum control and (B) 5 μM* c-myc-As-ODN *(original magnification ×200). Note: Increase in stippled nuclei in the antisense treated group. Figure 3C depicts the numerical analysis of nuclear labeling in serum control and* c-myc-ODN *treated groups.*

data for all four groups is shown in Figure 3C. The results showed a 30% decrease in dense nuclear labeling in the *c-myc-As-ODN* treated group and 30% increase in the stippled nuclei compared to the 2.5% serum control, whereas there was no significant decrease in dense labeling of nuclei or increase in the stippled nuclei in the sense oligonucleotide or nonsense oligonucleotide groups as compared to the 2.5% serum control group. These results confirm the specificity of the inhibitory effects of *c-myc-As-ODN*.

DNA flow cytometry analysis was done to determine DNA content of cells in the various oligonucleotide treated groups. The analysis showed a steady increase in less than 2N DNA content after *c-myc-As-ODN* treatment, suggesting a significant increase in the percentage of dead cells. A fivefold increase in less than 2N DNA content was noted after 4 days of 5 μM antisense oligonucleotide treatment, while no change was seen after serum, sense, or nonsense oligonucleotide treatment (Fig. 4). Analysis of the status of cell cycle indicated that there was no difference in percentage of cells entering different phases of cell cycle in control, *c-myc-As, sense,* and *nonsense ODN* treated groups suggesting that *c-myc-As-ODN* treatment did not inhibit progression of cell cycle.

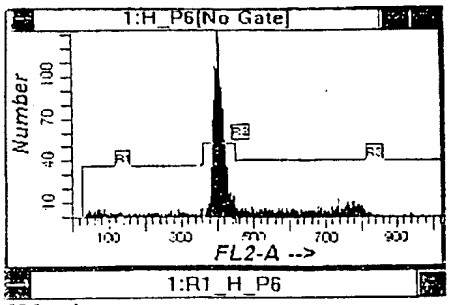
Comparison of our results on thymidine incorporation after *c-myc-ODN* treatment and release from isoleucine deprivation indicated that the thymidine incorporation was similar in *c-myc-As-ODN* treated cells (10 μM) and in cells at early S phase (Fig. 5).

COMMENT

The *c-myc* protooncogene is a cellular homologue of the avian myelocytic leukemia virus, which encodes for a nucleoprotein transcription factor that is strongly associated with cell proliferation, differentiation, and tumor formation.[22] The expression of *c-myc* is positively correlated with DNA synthesis and cell proliferation, and a direct role of *c-myc* in the cell cycle is suggested by the observation that quiescent cells enter the cell cycle and divide following microinjection of *c-myc* protein or transfection with the *c-myc* gene.[23] *C-myc* is overexpressed in a variety of cancers including prostate cancer.[7,8,24] *C-myc* amplification, rearrangement, and overexpression are features of prostate cancer cells,[25] hence these cell lines are appropriate models in which to study the effect of *c-myc-As-ODN* on androgen independent and dependent cell lines.

We targeted the *c-myc* protooncogene by using an oligonucleotide (15-mer) complementary to the translation initiation site on *c-myc* mRNA. Phosphorothioate derivative of oligonucleotides were chosen for our experiments because they possess greater stability in comparison to phosphodiester oligonucleotides.[15] To eliminate the effects of nucleases, *c-myc-ODNs* were added in serum free medium so that the cells could take up *ODNs* before serum was added. One may raise a question

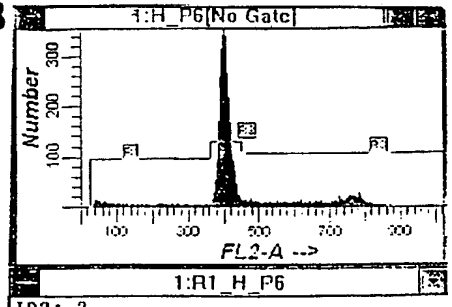
A



ID2: 1

	Percnt	%Gate	MeanX	MeanY
R1	6.48	6.48	172.68	0.00
R2	71.68	71.68	407.65	0.00
R3	21.84	21.84	666.55	0.00

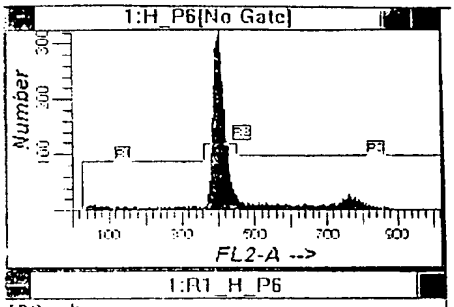
B



ID2: 2

	Percnt	%Gate	MeanX	MeanY
R1	5.83	5.83	158.53	0.00
R2	75.07	75.07	404.06	0.00
R3	19.11	19.11	662.54	0.00

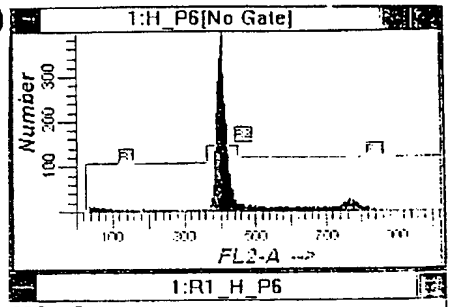
C



ID2: 4

	Percnt	%Gate	MeanX	MeanY
R1	5.57	5.57	182.87	0.00
R2	73.95	73.95	405.29	0.00
R3	20.48	20.48	662.95	0.00

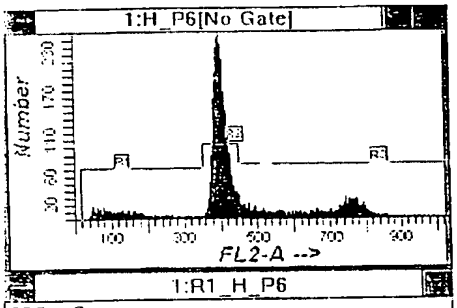
D



ID2: 3

	Percnt	%Gate	MeanX	MeanY
R1	5.50	5.50	141.58	0.00
R2	74.82	74.82	405.83	0.00
R3	19.68	19.68	663.75	0.00

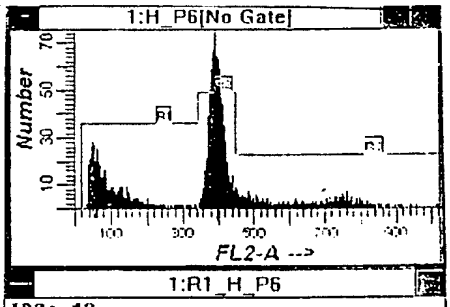
E



ID2: 9

	Percnt	%Gate	MeanX	MeanY
R1	9.09	9.09	141.38	0.00
R2	67.88	67.88	403.61	0.00
R3	23.05	23.05	664.80	0.00

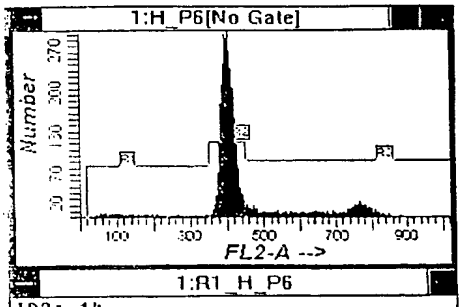
F



ID2: 12

	Percnt	%Gate	MeanX	MeanY
R1	26.80	26.80	92.74	0.00
R2	58.50	58.50	397.15	0.00
R3	14.70	14.70	637.89	0.00

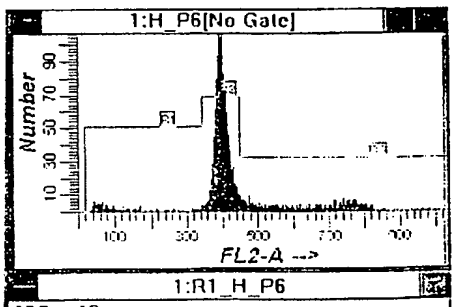
G



ID2: 14

	Percnt	%Gate	MeanX	MeanY
R1	3.71	3.71	149.42	0.00
R2	76.67	76.67	405.31	0.00
R3	19.63	19.63	684.07	0.00

H



ID2: 13

	Percnt	%Gate	MeanX	MeanY
R1	4.64	4.64	108.60	0.00
R2	78.22	78.22	399.91	0.00
R3	17.14	17.14	667.67	0.00

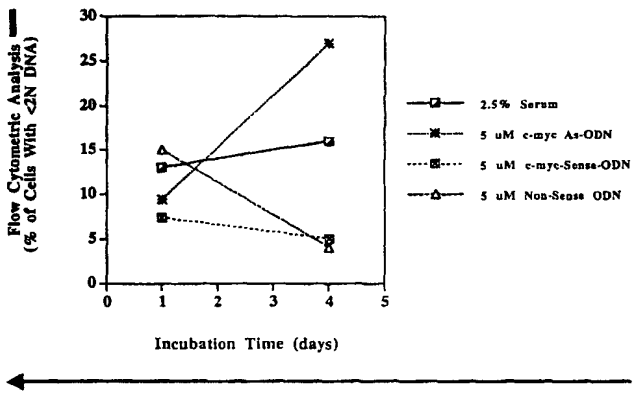


FIGURE 4. *A flow cytometric analysis of LNCaP cells treated with* c-myc-As, *sense and nonsense oligonucleotides. (A–D) 0 hour, (E–H) 96 hour, (A, E) 2.5% serum, (B, F)* c-myc-As-ODN, *(C, G)* c-myc-nonsense-ODN, *(D, H)* c-myc-sense-ODN. *Note an increase in the less than 2N DNA content spike at 4 days in the antisense oligonucleotide treated group (F) as compared to the sense and nonsense oligonucleotide treated groups. (I) Numerical analysis of the data.*

regarding the in vivo condition in which circulating blood must be taken into account. The current gene therapy technology involves packaging the gene or *ODNs* into retroviruses or liposomes for inoculation into the cancer cells ex vivo before administering into the individual, thereby ensuring the protection of the gene(s) in question. Therefore, the experimental design that we have adopted is appropriate in that it avoids any contact with enzymes that may destroy the *ODNs* or prior exposure to growth factors in the serum that may allow the cells to cycle through even after the addition of *As-ODNs*.

Our results indicate that cell growth and viability are decreased significantly in a dose- and time-dependent fashion after *c-myc-As-ODN* treatment (Fig. 1). Our [^{3}H]thymidine incorporation experiments and autoradiographic detection of [^{3}H]thymidine nuclear labeling confirmed the decrease in DNA synthesis (Fig. 2 and 3B) with 5 μM antisense oligonucleotide but no significant decrease in the 5 μM of sense or nonsense oligonucleotide treated groups. Inhibition of cell proliferation by *c-myc* antisense oligonucleotide for the mRNA translational initiation start site has been demonstrated for a number of other cell lines.[15,26,27] Data from flow cytometric analysis revealed an increase in less than 2N DNA content in the antisense oligonucleotide treated group indicative of DNA fragmentation and cell death.

Comparison of thymidine incorporation by LNCaP cells after *c-myc-As-ODN* treatment with that during the progression of cells from G1 to S phase indicate that DNA synthesis in *c-myc-As-ODN* treated cells was comparable to that observed in cells at early S phase. *C-myc-As-ODN* treatment did not arrest the cell cycle, since the percentage of cells entering different phases of the cell cycle was the same in all groups. It is likely that sustained decline in *c-myc* gene expression contributed to the decline in cell proliferation and viability. Similar results have been reported for HL-60 cells subjected to *c-myc-As-ODN* treatment.[28]

LNCaP cells are androgen dependent, and androgen withdrawal inhibits proliferation and survival of these cells initially. However, prolonged withdrawal of androgen makes these cells capable of growing in an androgen-independent manner.[29] Our preliminary results from *c-myc-As-ODN* treatment of two androgen-independent human cell lines (PC3 and DU145) suggest that *c-myc-As-ODN* treatment significantly affects the proliferation and survival of these androgen-independent cells. Our results also indicate that there is a difference in the degree of response by these various cell lines to *c-myc-As* treatment. For example, PC3 and LNCaP cells were more affected by the treatment in comparison to DU145. Nevertheless, inhibition of *c-myc* gene expression by using antisense oligonu-

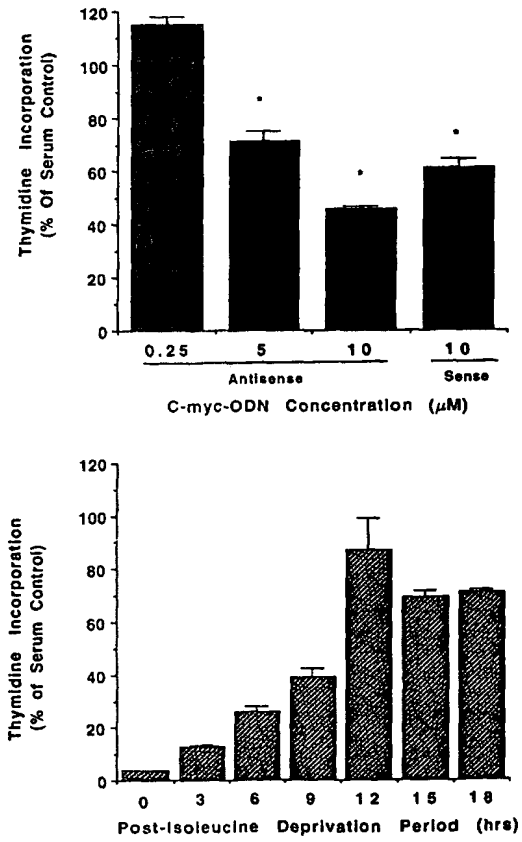


FIGURE 5. *Thymidine incorporation into DNA by LNCaP cells after isoleucine or* c-myc-ODN *treatments. The values are mean ± SE from six determinations (*P <0.05).*

cleotide may therefore be an effective method of treatment of both androgen-independent and -dependent prostate cancer cells. Currently, studies are underway to investigate the effects of androgen withdrawal plus *c-myc-As* treatment for an effective therapy of androgen-dependent cells.

Although our flow cytometry data indicate cell death, we do not know the mode of cell death in our experiments. However, antisense oligonucleotide treatment has been shown to decrease *c-myc* gene expression, and sustained decline in *c-myc* gene expression has been suggested to cause apoptosis.[28] Currently, studies are in progress to identify the mode of cell death and whether it is due to a decline in *c-myc* protein level or an alteration in the transcriptional regulation. A number of studies using various antisense oligonucleotides have shown hybridization of antisense nucleotides to respective mRNAs. These studies have reported involvement of hybridization mechanism at the translational level in the decrease of gene expression, resulting in reduced cell proliferation. Other studies, however, have challenged this hybridization mechanism by demonstrating a similar inhibitory response to nonantisense sequence. The nonhybridization mechanisms that may be involved in antisense oligonucleotide action are (a) the presence of four contiguous guanosine residues in the oligonucleotide sequence[30]; (b) nonspecific intracellular DNA-RNA hybrids[30]; and (c) nonspecific binding of antisense to proteins, such as CD4, gp120, or HIV-1 reverse transcriptase.[31]

The decrease in cell proliferation after 10 μM sense oligonucleotide treatment may be due to nonspecific inhibition independent of antisense mechanism since at lower concentrations the inhibitory effect was specific to the antisense oligonucleotide treated group. A similar decrease in cell proliferation has been shown in small cell lung cancer cells after *L-myc* sense oligonucleotide treatment.[15] Alternatively, it could also be due to an over-expression of *c-myc* in a cell line that has already up-regulated *c-myc* expression. Over-regulation of *c-myc* expression has been shown to inhibit proliferation and cause cell death.[28] That we did not find any decrease in less than 2N DNA content at lower concentrations of *c-myc* sense suggests that the decrease in cell proliferation in this cell line could be due to over-expression following *c-myc* sense treatment.

In summary, our results clearly indicate an inhibitory effect of *c-myc-As-ODN* treatment on prostate cancer cells consistent with previous reports for other cell lines.[7,10,15,27,28] This is the first in vitro report of an antisense DNA experiment targeting the *c-myc* gene in androgen-dependent and androgen-independent prostate cancer cell lines. At the current time, studies are in progress to investigate the mechanism by which *c-myc-As-ODN* inhibits cell survival and proliferation.

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Antisense Inhibition of 5-Hydroxytryptamine$_{2a}$ Receptor Induces an Antidepressant-Like Effect in Mice

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SUMMARY

Treatment with different antidepressants is invariably accompanied by the down-regulation of the 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) receptor. To determine whether receptor down-regulation is an essential part of antidepressant action, we manipulated levels of the 5-HT$_{2A}$ receptor by using a nonpharmacological approach. Here, we report that down-regulation of the 5-HT$_{2A}$ receptor by intracerebroventricular injection of antisense oligonucleotides resulted in an antidepressant-like effect in mice. Animals with 5-HT$_{2A}$ receptor deficiency showed less immobility in the Porsolt's forced swim test, a well established animal model that is used to identify drugs with an antidepressant effect. The overall locomotor activity of the receptor-deficient animals was not altered, demonstrating the specificity of the behavioral change in the Porsolt's forced swim test. Reduced immobility in this test was accompanied by a greater c-Fos response in piriform cortex. Because 5-HT$_{2A}$ receptors have been localized on γ-aminobutyric acid interneurons, the inhibitory activity of these neurons may be impaired at low receptor levels, leading to a greater c-Fos response in the piriform cortex and increased mobility in the Porsolt's forced swim test. These experiments demonstrate that down-regulation of the 5-HT$_{2A}$ receptor alone is sufficient to achieve an antidepressant-like effect in mice and suggest that receptor down-regulation may be an essential part of the antidepressant drug action.

A variety of different compounds have been found to have antidepressant activity. Although the pharmacological actions of these antidepressants are prompt, the clinical effects require weeks or even months to become manifest (see review in Ref. 1). This delayed response suggests that the development of antidepressant effect requires a plastic change in brain initiated by the drug treatment. A representative change, induced by long term antidepressant treatment, is the modulation of the 5-HT$_{2A}$ receptor. Virtually all antidepressants down-regulate the level of 5-HT$_{2A}$ receptor, and this down-regulation is temporally correlated with the onset of clinical efficacy (2–5). Antidepressants that down-regulate the 5-HT$_{2A}$ receptor include tricyclics, SSRIs, monoamine oxidase inhibitors, and atypical antidepressants such as mianserin (3). Tricyclic antidepressants block both norepinephrine and 5-HT uptake; SSRIs inhibit 5-HT transport; and monoamine oxidase inhibitors prevent the inactivation of monoamines. These drugs can be considered indirect agonists because they increase the availability of monoamines, especially 5-HT, in synaptic cleft. Increased levels of 5-HT in turn may initiate receptor down-regulation (6, 7). It is paradoxical that mianserin, a 5-HT$_{2A/2C}$ receptor antagonist with an antidepressant effect also elicits receptor down-regulation (2) because chronic treatment with antagonists generally induces disuse supersensitivity, a state characterized by an increase in receptor density (8). Nevertheless, the extent of down-regulation with mianserin is comparable to that achieved by tricyclics and SSRIs (2, 9).

Because treatment with different antidepressants modulates the level of 5-HT$_{2A}$ receptor, it is intriguing to hypothesize that receptor down-regulation is involved in or even required for the development of antidepressant effect. If receptor down-regulation is indeed an essential part of the antidepressant drug action, drugs with selectivity to the 5-HT$_{2A}$ receptor could be used to relieve certain symptoms of depression. On the other hand, if receptor down-regulation is not directly linked to the therapeutic effect, drugs that avoid receptor effect may be more selective in the treatment of depression. To determine whether receptor down-regulation is an essential part of antidepressant drug action, the 5-HT$_{2A}$

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; GABA, γ-aminobutyric acid; SSRI, selective serotonin reuptake inhibitor; AS, antisense; MS, mismatched; aCSF, artificial cerebrospinal fluid; LSD, lysergic acid diethylamide; FST, Porsolt's forced swim test; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; PTZ, pentylenetetrazol; PCR, polymerase chain reaction.

receptor was down-regulated directly and specifically by intracerebroventricular injection of a receptor-specific AS oligonucleotide. In contrast to the pharmacological approach, which blocks receptor function by antagonists, the AS approach provides down-regulation of the 5-HT$_{2A}$ receptor number that is more analogous to the effect of chronic antidepressant treatment. Moreover, selectivity of AS oligonucleotides is greater than that of most receptor antagonists. AS oligonucleotides can specifically recognize receptor mRNA, facilitate its degradation, or interfere with its translation, resulting in a reduced receptor level (10). Here, we report that AS-induced down-regulation of the 5-HT$_{2A}$ receptor results in an antidepressant-like effect in mice. This result suggests that down-regulation of 5-HT$_{2A}$ receptor alone may have some therapeutic benefit in the treatment of mood disorders.

Materials and Methods

Oligonucleotide treatment. Balb/C mice (6–8 weeks old) were injected alternatively into the left and right lateral ventricles every 12 hr for 4 days with 10 μg of either AS (5′-AGACACTTCTGTTATAGA-3′) or MS (5′-AGTCACTGCTGTTATGGA-3′) oligonucleotide in 5 μl of aCSF. The oligonucleotides corresponded to the 5′-translated region of the 5-HT$_{2A}$ receptor. The MS oligonucleotide differed in three positions from the AS oligonucleotide. A control group of mice was injected with 5 μl of aCSF. All behavioral tests were performed on the fifth day.

For localization of the oligonucleotide, animals (three mice) were injected with a biotin-labeled AS oligonucleotide five times over 2.5 days. Control animals were injected with either aCSF (two mice), unlabeled oligonucleotide (two mice) or 0.2 mM biotin in aCSF (two mice). Twelve hours after the last oligonucleotide injection, animals were anesthetized with pentobarbital sodium (Nembutal Sodium, 150 mg/kg; Abbot Laboratories, King-of-Prussia, PA) and perfused intracardially with 4% paraformaldehyde. Brain sections (40 μm) were obtained on a freezing microtome, treated with 0.5% H_2O_2 to remove endogenous peroxidase activity, and incubated with the ABC complex of the Vector Elite Kit (1:100) (Vector Laboratories, Burlingame, CA) as well as with substrate, according to the manufacturer's instructions.

Autoradiography. 5-HT$_{2A}$ receptor binding was carried out on brain sections using ^{125}I-labeled LSD (50 pM), according to a published procedure (11). 5-HT$_{2A}$-nonspecific binding (≈30% of total) was determined in the presence of 200 nM spiperone. Spiperone blocks both the 5-HT$_{2A}$ and dopamine D$_2$ receptors. The D$_2$ receptor component of the total binding was 19% in cortical layers and 26% in striatum, as determined by competitive displacement on parallel sections with increasing concentrations of haloperidol (IC$_{50}$ = 0.4 nM). The 5-HT$_{2A}$ receptor-specific component of binding was calculated from the total binding by subtracting nonspecific and D$_2$ receptor-specific binding. Sections were exposed overnight to Hyperfilm (Amersham, Arlington Heights, IL), and computed densitometry was performed with the NIH Image program. Quantification was based on a series of ^{125}I-autoradiographic internal standards (Amersham).

5-HT$_{2C}$ receptor binding was measured in the choroid plexus, a region abundant in these receptors, by using ^{125}I-labeled LSD in the presence of spiperone. Because 200 nM spiperone displaced 5-HT$_{2A}$ receptor binding (IC$_{50}$ = 8 nM) as well as D$_2$ receptor binding, the remaining signal was solely due to 5-HT$_{2C}$ receptor binding. Nonspecific binding was measured in the presence of 2 μM mianserin. Mianserin displaces 5-HT$_{2A}$, 5-HT$_{2C}$, and D$_2$ receptor binding. Sections were exposed for 8 days.

c-Fos immunohistochemistry. The assay was performed as described previously (12). Two hours after FST, animals were anesthetized with pentobarbital sodium (150 mg/kg) and perfused intracardially with 4% paraformaldehyde. Free floating sections (40 μm) were incubated with a c-Fos antiserum (Fos and related antigens, 1:8000 dilution; Cambridge Research Biochemicals, Northwich, UK). The antigen was visualized with the ABC Vector Elite Kit. c-Fos immunoreactive nuclei were counted on parallel slides with a bright-field microscope at 10× magnification. c-Fos-positive nuclei were counted for the whole frontal piriform cortex, without correction, in three successive sections per mouse brain. The numbers of animals involved in this test were four for aCSF, five for MS, and five for AS.

Quantitative RT-PCR. RT-PCR was performed essentially as described previously (13). Briefly, total RNA was prepared from frontal cortex by using TRIZOL Reagent (GIBCO BRL, Gaithersburg, MD). Then, 9 μg of total RNA was incubated with 1 unit of RNase-free DNase I (GIBCO BRL) in the presence of 20 units of RNasin ribonuclease inhibitor (Promega, Madison, WI) to remove any remaining genomic DNA. The DNA-free RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) using a primer corresponding to bases −381 to −399 in the 5′-untranslated region of the 5-HT$_{2A}$ receptor (5′-AAACAGCATGAGATCCAA-3′). Reverse-transcribed cDNA, corresponding to 62, 31, and 15.5 ng of total RNA, was used for PCR amplification. Primers complementary to bases −381 to −399 (see sequence above) and −781 to −761 (5′-CTCAAAGAGAGGGGATTCCACA-3′) yielded a 400-bp product. During PCR, [^{32}P]dATP was incorporated into the product to allow for quantification in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Behavioral studies. Headshakes were registered during a 10-min period at 30 min after a 2.5 mg/kg intraperitoneal dose of DOI. In FST, mice were forced to swim in a 8-in wide water-filled cylinder, essentially as described by Porsolt *et al.* (14). In this test, immobility of the mice is measured in blocks of 2 min for a total of 6 min. All animals were naive and submitted only once to each test. The open field apparatus consisted of a 15- × 21-inch black box divided into six (2- × 3-inch) even-sized rectangles. The number of crosses in open field was recorded for 10 min. The elevated plus maze was performed according to standard procedures (15) using a cross-maze with 12- × 2-inch arms. The number of entries into and time spent in the open arms as well as the total number of entries were recorded in a 10-min test. Care of all mice in this work was in accordance with institutional guidelines.

Seizure susceptibility measured by PTZ test. Mice were injected with 85 mg/kg PTZ intraperitoneally, and seizure events were videotaped during an observation period of 20 min. Mice were scored for the sequence of four seizure behaviors produced by the drug (i.e., myoclonic jerks, clonic convulsions, tonic phase, and death). A human-guided computer-assisted scoring system was used to evaluate seizures (16). The timing of the seizure behaviors, as well as their duration after the PTZ injection, was recorded. Myoclonus was defined as a single movement of the mouse that involved a downward motion of the head, combined with a single jerk of the body, and a brief upward extension of the tail. Clonus usually was the second seizure behavior to occur chronologically after PTZ injection; it was defined as rapidly repetitive jerks of the mouse that involved the entire body such that the mouse would fall to the side. The tonic stage of the seizure, when reached, was defined as a slow hindlimb extension. In these mice, the tonic stage was invariably followed by death.

Statistical analysis. Performance in behavioral studies, absorbance of autoradiographs, and c-Fos positive nuclei were compared between groups using a one-way analysis of variance followed by Scheffé's *post hoc* test. Statistical difference was determined at a level of $p < 0.05$. For autoradiography and c-Fos quantification, multiple successive sections were quantified in each sector sampled, and the average for this sector was calculated in each mouse. The results of the PTZ test were calculated by nonparametric analysis of variance followed by the χ^2 test.

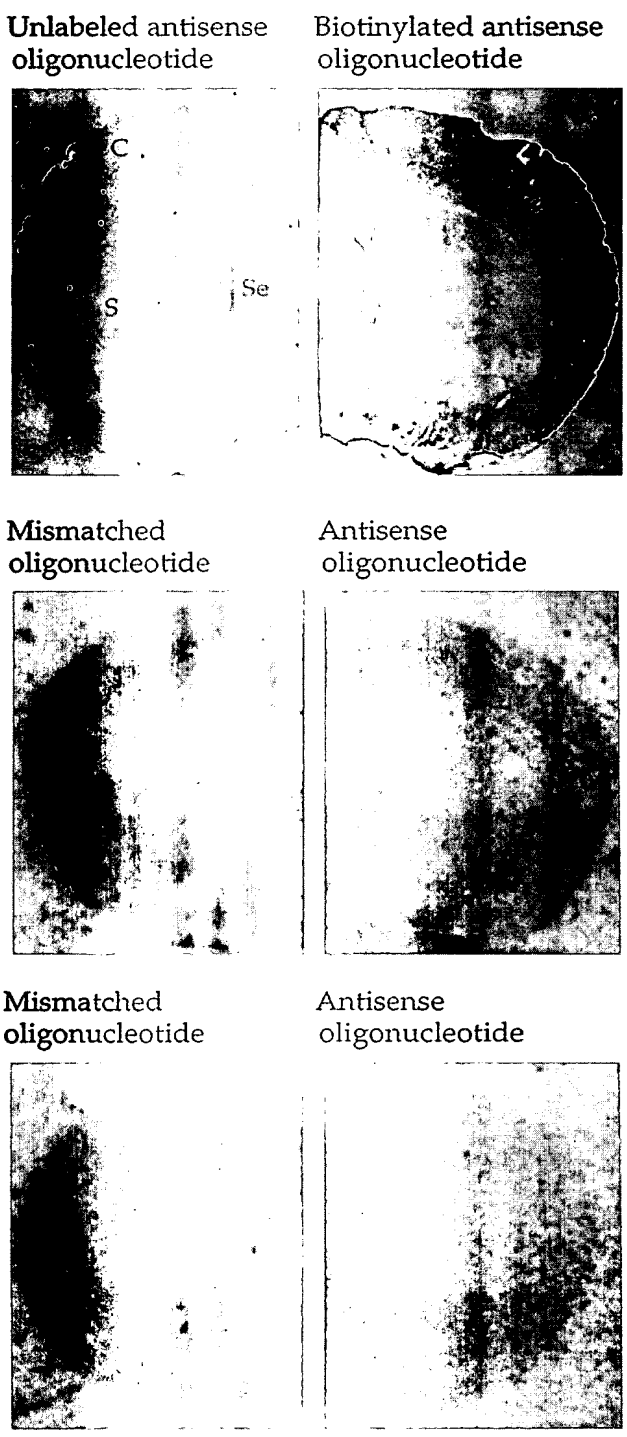

Fig. 1. Intracerebroventricular injection of oligonucleotides into mouse brain. A, Biotin-labeled AS oligonucleotide administered alternatively to the left and right lateral ventricles (*right*) is taken up preferentially by the cortex (*C*). The oligonucleotide was visualized by a peroxidase enzymatic reaction. Subcortical regions such as striatum (*S*) and septum (*Se*) accumulated less oligonucleotide compared with the cortex. Thalamus and hypothalamus are not visible at the anatomic level displayed but also contained low levels of the administered oligonucleotide. No specific staining was found in brains of animals injected with unlabeled oligonucleotide (*left*). B, AS oligonucleotide injection down-regulated cortical 5-HT_{2A} receptors (*right*), as measured by receptor autoradiography using ^{125}I-labeled LSD. Results of the measurements on regional 5-HT_{2A} receptor concentration are displayed in Fig. 2. C, Nonspecific binding in the presence of 200 nM spiperone.

Results

AS oligonucleotide treatment selectively down-regulates 5-HT_{2A} receptor levels and attenuates receptor function. In the brain, 5-HT_{2A} receptors are found mostly in the cortical layers and striatum. Because the most dramatic antidepressant-induced receptor down-regulation occurs in the cortex (3), we tested whether oligonucleotides injected into the lateral ventricles reached the cortical layers. A biotin-labeled oligonucleotide was repeatedly injected (intracerebroventricular injections every 12 hr for 2 days), and its localization was visualized by peroxidase enzymatic staining (Fig. 1A). A predominantly cortical distribution was observed, presumably due to the transport of the oligonucleotide to the subarachnoidal space that overlies the entire cortex and provides a large surface for uptake. Subcortical regions such as striatum or thalamus accumulated fewer oligonucleotides. Intermediate levels were found along the midline, in the septum ,and in the hypothalamus. The highest levels of oligonucleotides were found in the choroid plexus, presumably because it was in a direct contact with the oligonucleotides injected into the lateral ventricles. Mice injected with unlabeled oligonucleotide (Fig. 1A), aCSF, or biotin alone (data not shown) did not show any detectable staining.

Repeated intracerebroventricular injection of a 18-mer AS oligodeoxynucleotide, corresponding to the 5′-translated region of the 5-HT_{2A} receptor (10 μg in 5 μl of aCSF every 12 hr for 4 days), resulted in 47%, 46%, and 48% decreases in specific binding in frontal, parietal, and piriform cortices, as measured by autoradiography with ^{125}I-labeled LSD (Figs. 1B and 2A). Because the central nervous system has a low nuclease activity, unmodified phosphodiester oligonucleotides were used, avoiding the toxicity inherent to the more stable phosphorothioate oligonucleotides. Injection of neither MS oligonucleotides (three mismatched bases in the AS sequence) nor aCSF altered receptor binding. In the striatum, the down-regulation by the AS oligonucleotide was only 30%, which is in agreement with the lower level of oligonucleotide accumulation in this region (Figs. 1A and 2A). Other regions, such as thalamus, hypothalamus, and hippocampus, showed a low level of specific binding, and the effect of AS oligonucleotide in these regions could not be reproducibly measured.

To evaluate the specificity of the AS oligonucleotide for the 5-HT_{2A} receptor, we tested its effect on the 5-HT_{2C} receptor. The 5-HT_{2A} and 5-HT_{2C} receptors are closely related; their ligand binding properties, coupling, and amino acid sequence are similar (17). However, their nucleotide sequences are not entirely identical, and the AS oligonucleotide was selected so it would not interact with the 5-HT_{2C} receptor sequence. 5-HT_{2C} receptor binding was measured in the choroid plexus, a region abundant in these receptors. Because the choroid plexus contained the highest level of oligonucleotide, it was an ideal brain region in which to assess whether the 5-HT_{2A} receptor-specific oligonucleotide can alter 5-HT_{2C} receptor binding. As Fig. 2A shows, no attenuation of the 5-HT_{2C} receptor was measured after repeated oligonucleotide injection; thus, the effect of AS oligonucleotide was selective for the 5-HT_{2A} receptor.

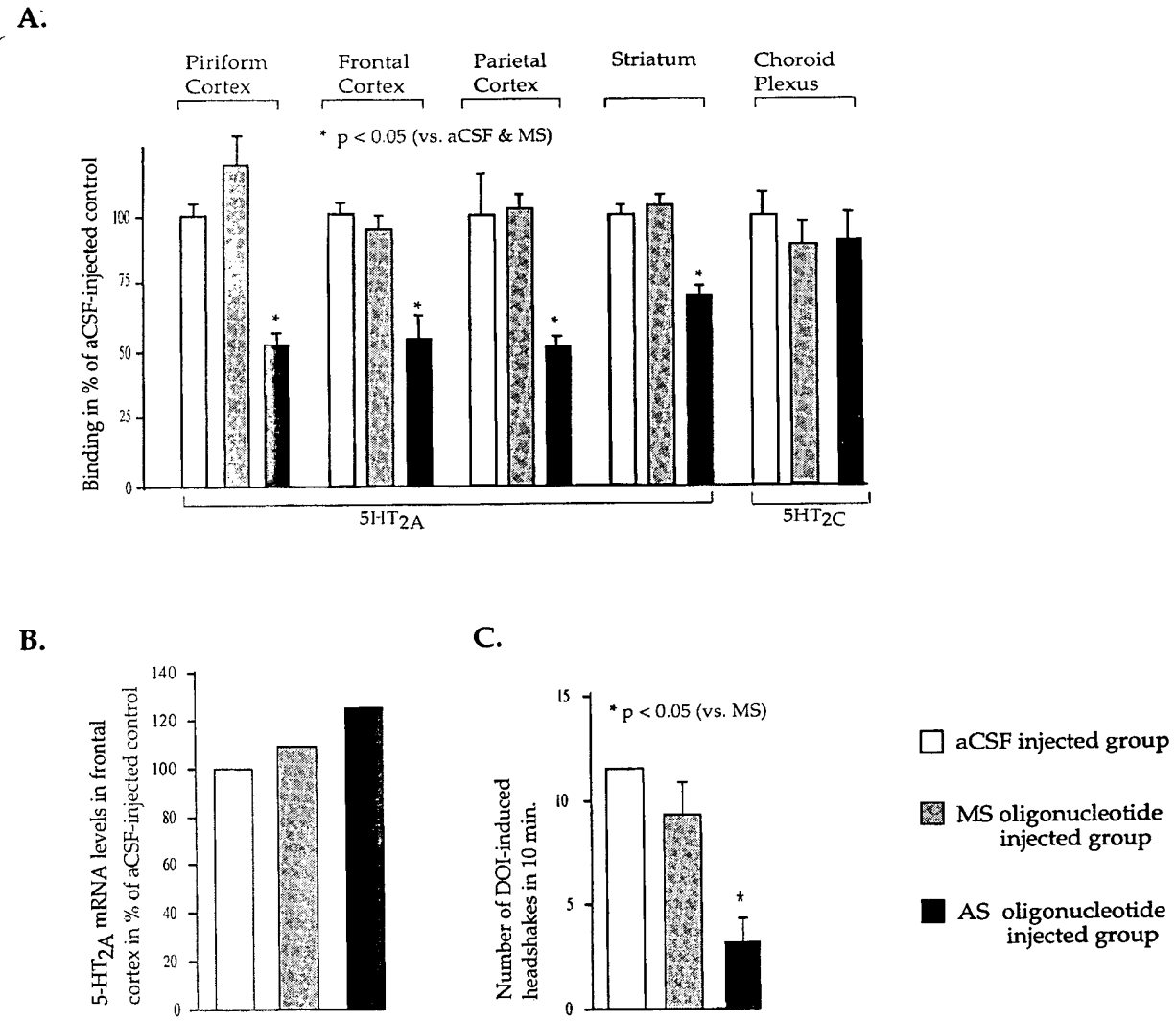

Fig. 2. Receptor down-regulation and functional receptor deficit in AS oligonucleotide-injected animals. A, Receptor down-regulation in cortical regions of AS oligonucleotide-injected animals measured by receptor autoradiography. Striatum showed a less profound but statistically significant receptor down-regulation. The level of the closely related 5-HT$_{2C}$ receptor was not significantly altered, demonstrating the selectivity of the down-regulation for the 5-HT$_{2A}$ receptor. Values (mean ± standard error) are shown for aCSF (five animals), MS (six animals), and AS (six animals) oligonucleotide-injected animals. B, The 5-HT$_{2A}$ receptor mRNA level was not affected by AS oligonucleotide injection in cortex as measured by reverse transcription PCR from total RNA derived from frontal cortex. Values are mean of four RNA samples isolated from two animals in each group. C, Down-regulation of the 5-HT$_{2A}$ receptor resulted in a functional receptor deficit. The number of DOI-induced headshakes, mediated by 5-HT$_{2A}$ receptors, was significantly decreased in AS oligonucleotide-injected animals compared with MS oligonucleotide- and aCSF-injected mice. Values (mean ± standard error) are shown for aCSF (two animals), MS (six animals), and AS (four animals) oligonucleotide-injected animals.

Interestingly, attenuation of receptor binding by AS oligonucleotide injection in the frontal cortex was not accompanied by the down-regulation of 5-HT$_{2A}$ receptor mRNA (Fig. 2B), suggesting a post-transcriptional/translational mechanism for the AS oligonucleotide action. To determine whether the receptor down-regulation was accompanied by a functional attenuation, we measured the number of DOI-induced headshakes (18). Headshakes induced by DOI are thought to originate in the brainstem and be facilitated by diencephalic structures (19), but alterations in 5-HT$_{2A}$ receptor density in frontal cortex can also modify the headshake response (20, 21). Headshakes induced by DOI were significantly decreased in AS oligonucleotide-injected animals compared with control mice (Fig. 2C). Although the decreased headshake response cannot be attributed to a specific pool of receptors, it nevertheless demonstrated a functional 5-HT$_{2A}$ receptor deficit in the brain of AS oligonucleotide-injected mice.

Taken together, these results demonstrated that repeated intracerebroventricular injection of AS oligonucleotide can selectively decrease 5-HT$_{2A}$ receptor binding, mostly in the cortex, leading to the attenuation of receptor function.

Receptor down-regulation increases mobility in FST. FST is an animal model of depression that is used routinely for preclinical testing of antidepressants (14). FST is sensitive to tricyclic antidepressants, monoamine oxidase

inhibitors, and atypical antidepressants. FST is also sensitive to SSRIs in both mice and rats (22); however, some SSRIs are less active and sometimes even inactive in rats (23). The test is based on the observation that when forced to swim in a restricted space from which there is no escape, mice will gradually cease attempts to escape and become immobile. It was suggested that immobility reflects a state of despair that can be reduced by a variety of drugs and treatments that are therapeutically effective in depression (22). As Fig. 3A shows, immobility of AS oligonucleotide-injected animals was reduced significantly compared with control animals (aCSF and MS oligonucleotide-injected animals). The test was validated by using the atypical antidepressant mianserin at the highest nonsedative dose of 3 mg/kg. As expected, mianserin had an anti-immobility effect in FST comparable to that induced by AS oligonucleotide injection (Fig. 3B). However, mianserin can block both the 5-HT_{2A} and 5-HT_{2C} receptors. On the other hand, the selective 5-HT_{2A}

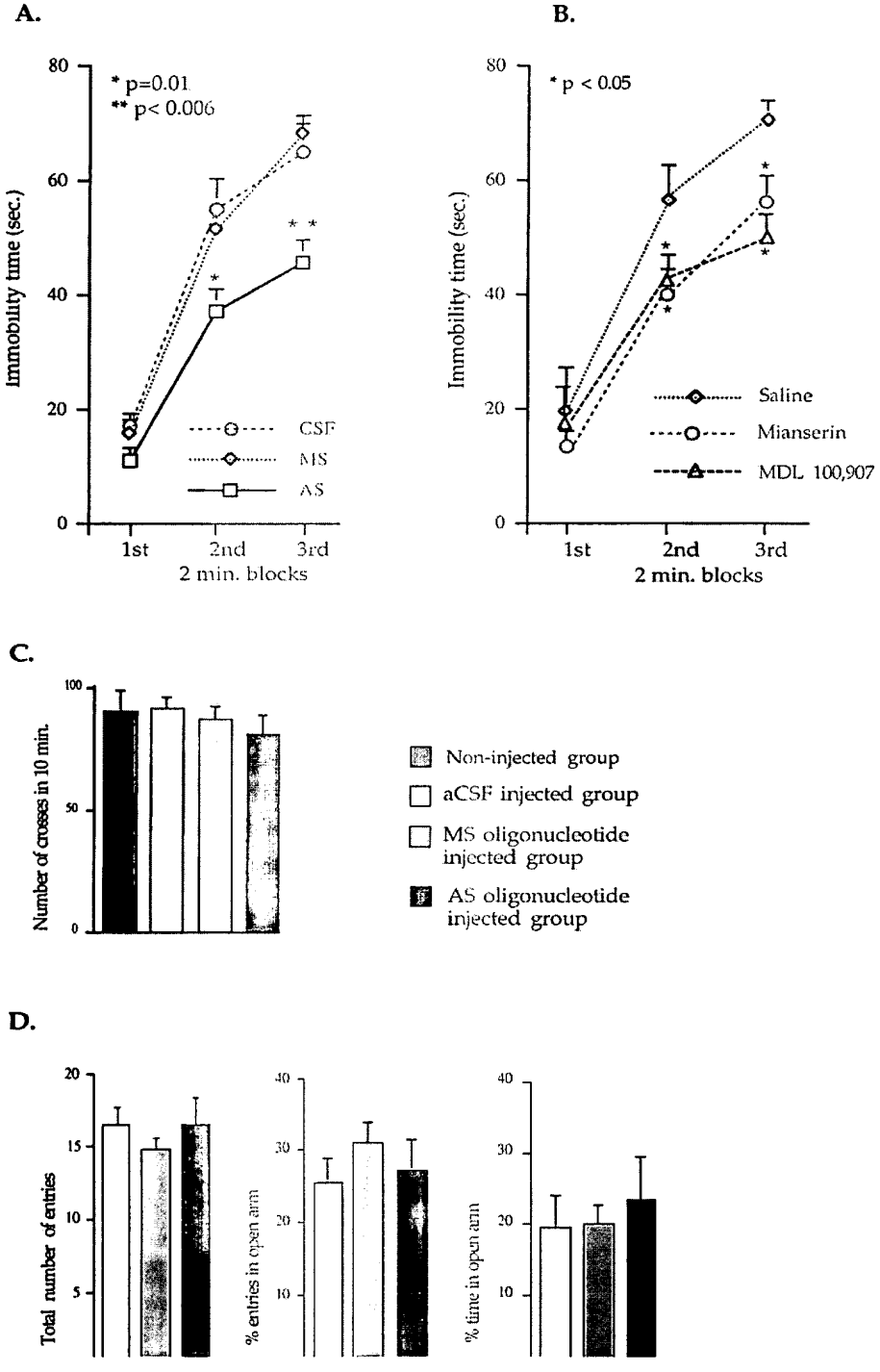

Fig. 3. Behavioral characterization of mice with a deficit in cortical 5-HT_{2A} receptor. A, FST. Animals exposed to swim stress show an intense escape-directed behavior in the first block of the test. aCSF and MS oligonucleotide-injected animals subsequently display a gradually diminishing mobility throughout the second and third blocks of the test. AS oligonucleotide-injected animals showed less immobility. Values (mean ± standard error) are shown for aCSF (13 animals), MS (20 animals), and AS (22 animals) oligonucleotide-injected animals. B, Mianserin (3 mg/kg; 10 animals) and MDL 100907 (0.15 mg/kg; 7 animals) reproduce the AS oligonucleotide-induced behavioral effect in FST. Control animals were injected with saline (10 animals). C, Locomotor activity of AS oligonucleotide-injected animals is not altered in the open field (noninjected, 8 animals; aCSF, 8 animals; MS, 8 animals; AS, 9 animals). D, Elevated maze. AS-treated animals did not differ from control groups in percentage of time and entries in the open arms as well as in total number of entries in all arms of the maze (aCSF, 14 animals; MS, 17 animals; AS, 19 animals).

receptor antagonist MDL 100,907 (24) was also effective in FST, demonstrating that blockade of the 5-HT$_{2A}$ receptor can lead to an anti-immobility effect (Fig. 3B).

The anti-immobility effect in AS oligonucleotide-injected animals was not due to increased basal locomotion because activity of these animals in open field was similar to that of control mice (Fig. 3C). In addition, receptor down-regulation had no detectable effect in the elevated maze (Fig. 3D). We conclude that a reduction in 5-HT$_{2A}$ receptor level is sufficient to increase mobility in FST.

Increased c-Fos response of AS oligonucleotide-injected animals in the piriform cortex. FST induces the expression of the immediate-early gene c-*fos*, indicating an extensive neuronal activation during swimming (25). Using immunohistochemistry directed toward the c-Fos protein and related antigens, we observed no difference between AS and MS oligonucleotide-injected animals in subcortical nuclei (lateral septal nucleus, bed nucleus of the stria terminalis, and hypothalamic and thalamic paraventricular nuclei; data not shown). However, a greater increase in immunoreactivity was found in the piriform cortex of AS oligonucleotide-injected animals compared with control animals [150 ± 11.7% of control (mean ± standard error), $p < 0.005$] (Fig. 4). The FST-induced c-Fos response was not significantly increased in other cortical regions of AS oligonucleotide-injected animals compared with control (MS- and aCSF-injected) mice (data not shown).

The increased c-Fos response in AS oligonucleotide-injected animals after the forced swim raised the possibility of a general hyperexcitability as a consequence of down-regulation of the 5-HT$_{2A}$ receptor. The overall excitability of the brain of AS oligonucleotide-injected mice was measured by using the seizure-inducing agent PTZ. PTZ seems to decrease the potency of GABA-mediated inhibition in brain (26) and, depending on the dosage, can produce myoclonic jerks, clonic convulsions, tonic seizures, and death in animals. The 85 mg/kg dose of PTZ used in this experiment was equally potent to produce myoclonic jerks and clonic convulsions in both AS and MS oligonucleotide-injected animals (Table 1). However, there was a tendency for more lethality among AS oligonucleotide-injected mice, albeit the difference between AS oligonucleotide-injected and control animals did not reach statistical significance. These results argue for no increased excitability in AS oligonucleotide-injected animals, at least not in brain regions involved in initiating PTZ seizures, such as the reticular formation and the anterior thalamus (27, 28). These data also suggest that increased neuronal activation in AS oligonucleotide-injected animals is restricted to certain regions that may correspond to areas rich in 5-HT$_{2A}$ receptor.

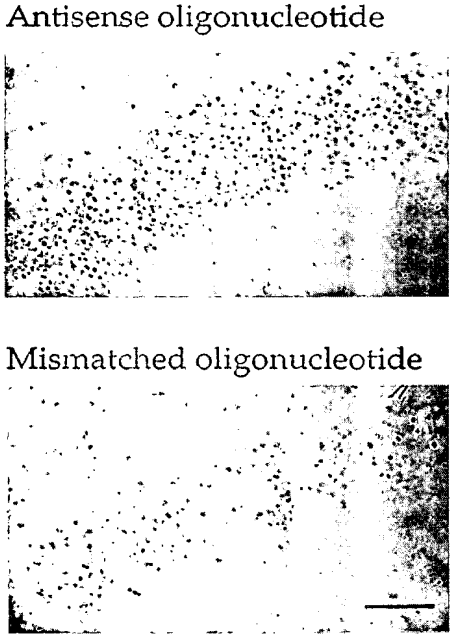

Fig. 4. Increased c-Fos response after FST in the piriform cortex of a mouse with a deficit in 5-HT$_{2A}$ receptors (*top*, AS oligonucleotide-injected animal) compared with a control (*bottom*, MS oligonucleotide-injected animal). *Bar*, 40 μm. Number of c-Fos-positive nuclei/0.01 mm^2 were 66 ± 5.6 for AS, 44.3 ± 3.4 for MS, and 48 ± 5.0 for aCSF.

Discussion

The down-regulation of the 5-HT$_{2A}$ receptor by virtually all antidepressants raised the possibility of receptor involvement in drug action. However, antidepressants down-regulate the 5-HT$_{2A}$ receptor by different mechanisms, and it has been difficult to determine whether the receptor down-regulating and therapeutic effect of antidepressants are linked. For example, fluoxetine, a 5-HT transporter blocker and effective antidepressant probably has no direct action on the receptor itself; rather, it is the elevated level of 5-HT that may down-regulate the receptor. In contrast, mianserin, a 5-HT$_{2A/2C}$ receptor antagonist and atypical antidepressant presumably acts on the receptor itself (29).

To study whether down-regulation of the receptor leads to an antidepressant-like effect, the level of 5-HT$_{2A}$ receptor was reduced by injecting AS oligonucleotides into the brain of mice. The antisense approach provided down-regulation of the 5-HT$_{2A}$ receptor that was analogous to the reduction in receptor number induced by chronic antidepressant treatment. Even the extent of the down-regulation of the receptor in the frontal cortex by AS oligonucleotide injection (≈47%, Figs. 1 and 2) was similar to that achieved by antidepressant treatment (up to 50%) (3, 30). The effect of AS oligonucleotide injection was selective for the 5-HT$_{2A}$ receptor because no attenuation of the 5-HT$_{2C}$ receptor was detectable (Fig. 2A). Receptor down-regulation was accompanied by a functional attenuation because DOI-induced headshakes were significantly decreased in AS oligonucleotide-injected animals (Fig. 2C).

As Fig. 3A demonstrates, down-regulating the level and attenuating the function of the 5-HT$_{2A}$ receptor by intracerebroventricular injection of a receptor-specific AS oligonucleotide resulted in a significant change in the behavioral response of mice. Receptor down-regulation inhibited immobility in FST, which is an indication of an antidepressant effect. The extent of anti-immobility effect in receptor-deficient animals was comparable to that induced by the atypical antidepressant mianserin (Fig. 3B). Clinically active antidepressants have an anti-immobility effect in FST without altering locomotor activity in open field. Likewise, no locomotor activation was seen in AS oligonucleotide-injected animals (Fig. 3C). Taken together, experiments involving AS oligonucleotide injections demonstrate that down-regulation of the

TABLE 1
Susceptibility of AS oligonucleotide-injected and control animals to PTZ-induced seizures

	aCSF (n = 8)	MS (n = 8)	AS (n = 8)
Animals displaying jerks (n)	7	8	7
Mean number of jerks in 20 min	50 ± 10	57 ± 2	47.4 ± 9.9
Time spent in jerks in 20 min (sec)	28.9 ± 10.8	39 ± 11	34.7 ± 15
Animals displaying clonus (n)	7	8	7
Mean number of clonus in 20 min	1.4 ± 0.3	2 ± 0.5	1.4 ± 0.9
Time spent in clonus in 20 min (sec)	15.3 ± 5.6	15.3 ± 4	13.4 ± 5.7
Animals displaying tonic seizures (n)	1	2	4[a,b]
Animals that died (n)	1	2	4[a,b]

[a] p = 0.10 versus aCSF; [b] p = 0.3 versus MS, χ^2 test.

receptor alone is sufficient to achieve an antidepressant-like effect in mice.

How does down-regulation of 5-HT_{2A} receptor inhibit immobility in FST? Although it is attractive to interpret immobility as a sign of despair and compare it with depression, there is no evidence to support this notion. Rather, behavior of mice in FST is more similar to a typical response to stress. Initially, stress activates a number of effector systems within the central nervous system that promote arousal and vigilance. However, when stress becomes chronic, the acute behavioral stress responses are gradually diminished, presumably because excessive stress reactions would be counterproductive (exhaustion to swim). The stress nature of FST is supported by the activation of the hypothalamic-pituitary-adrenal axis and the induction of the early-immediate gene c-*fos* after swimming (25, 31). Both AS and MS oligonucleotide-injected animals showed an ≈4-fold increase in plasma corticosterone levels 10 min after the initiation of FST (data not shown). Also, the temporal and spatial patterns of c-*fos* activation were very similar to that induced by stressful stimuli such as restrain (Ref. 25; see also Fig. 4). Therefore, the gradually developing immobility in FST can represent a containment process (32) rather than a despair. Whether the behavior in FST is a stress response or more of a despair-like reaction, the data presented here unequivocally demonstrate that it can be significantly altered by the 5-HT_{2A} receptor.

The anti-immobility effect of receptor down-regulation may be explained by the specific localization of the receptors in the central nervous system. 5-HT is present at the terminal area of fine 5-HT immunoreactive axons in the cortex that arise from the dorsal raphe nucleus (33). In these areas, 5-HT_{2A} receptors are frequently found on interneurons. Based on morphology and electrophysiological properties, 5-HT_{2A} receptor-bearing interneurons are likely GABAergic cells (34–36). 5-HT_{2A} receptor-bearing interneurons form a dense band on layer III in the piriform cortex (34) and could provide an inhibitory cortical input. Low 5-HT_{2A} receptor levels in AS oligonucleotide-injected animals may lead to increased pyramidal activity due to less activation of the inhibitory GABAergic interneurons during FST. In turn, the increased neuronal activity could result in an anti-immobility effect by augmenting neuronal pathways that mediate the behavioral stress responses such as the escape-directed behavior.

Although GABAergic interneurons expressing 5-HT_{2A} receptors are also localized in the frontal/parietal cortex, the overall number of these cells represents a relatively small portion of the total cell number in these regions (35). Moreover, the 5-HT_{2A} receptor is also expressed in pyramidal cells in the frontal/parietal cortex, which could counteract the effect on GABAergic function by activating pyramidal cells directly. Therefore, AS down-regulation of the receptor in neocortex could have less impact on the overall neuronal activity, and this situation would render it undetectable by c-Fos immunostaining. The localized nature of increased neuronal activity is also supported by the lack of overall brain hyperexcitability shown in the PTZ test (Table 1). Although not revealed by our immunostaining experiments, a 5-HT_{2A} receptor-mediated tonic inhibition may exist in the medial prefrontal cortex. Schmidt *et al.* (24) showed that blocking 5-HT_{2A} receptors by the selective antagonist MDL 100,907 results in an increase in dopamine efflux in rats. These results raise the possibility that the behavioral effects of the AS oligonucleotide injection may also be due to an increased dopamine release during forced swim. Taken together, the data presented here support the hypothesis that down-regulation of 5-HT_{2A} receptors disinhibits piriform cortex and perhaps other receptor-rich cortical areas in mice that could lead to a state of increased psychomotor activity, visible as anti-immobility effect in FST.

The antidepressant-like effect induced by AS oligonucleotide injection in mice is consistent with the beneficial effect of pharmacological blockade of the 5-HT_{2A} receptor in dysthymic disorders. Studies with ritanserin, a 5-$HT_{2A/2C}$ receptor antagonist, showed that a group of patients with anxiety syndrome felt less tired and more energetic after treatment (37). This observation prompted further studies that showed a benefit of ritanserin in patients with dysthymic disorder characterized by anergy, lack of motivation, and depressive mood (37). It is tempting to speculate that block of 5-HT_{2A} receptors would increase psychomotor activity that could counterbalance the psychomotor retardation present in these patients. However, antidepressant action is certainly more complex than producing a state of psychomotor activation. Nevertheless, psychomotor activation could be an important part of antidepressant drug action. Indeed, sympathomimetic stimulants, such as amphetamine and amphetamine surrogates, are occasionally used as antidepressants.

Another disease characterized by psychomotor retardation is schizophrenia, which may also respond to the manipulation of the 5-HT_{2A} receptor (38). Indeed, the 5-$HT_{2A/2C}$ receptor antagonist ritanserin and the 5-HT_{2A}/D2 receptor antagonist clozapine showed a benefit in schizophrenia, in particular by ameliorating negative symptoms (39, 40).

Taken together, we propose that down-regulation of the 5-HT_{2A} receptor contributes to the beneficial effect of antidepressants by producing a state of increased psychomotor activity. Drugs with selectivity to the 5-HT_{2A} receptor could be used to relieve certain symptoms of depression. Recently,

MDL 100,907, a selective 5-HT$_{2A}$ receptor antagonist, has been developed (40). MDL 100,907, is currently in clinical trial for schizophrenia, but it would be interesting to test this compound in depression, too.

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